

**Ion transport physiology and its interaction with trace element
accumulation and toxicity in inanga (*Galaxias maculatus*)**

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Table of Contents

Acknowledgements.....	6
Abstract.....	7
List of tables & figures	9
Abbreviations.....	14
Chapter 1: Introduction	15
1.1 Background	16
1.2 Osmoregulation in freshwater fish	16
1.3 Trace Element toxicity.....	18
1.3.1 Sources of trace elements.....	18
1.3.2 Bioavailability	19
1.3.3 Trace element uptake	21
1.3.4 Toxic effects of Fe, Zn, and Cu	22
1.4 Biotic Ligand Model	25
1.5 Biomarkers	26
1.6 Approaches to studying trace element toxicity	27
1.7 Inanga.....	28
1.8 Synopsis.....	31
Chapter 2: General Methods	34
2.1 Animal collection & husbandry	35
2.2 Aquarium water	35
2.3 Euthanasia and dissections	37
2.4 Unidirectional ion flux.....	37
2.4.1 Ion influx	37
2.4.2 Na ⁺ Efflux.....	38
2.5 Caging exposures	39
2.6 Natural organic matter (NOM).....	40
2.7 Trace element analyses.....	40
2.7.1 Sample digestions	40
2.7.2 ICP-MS.....	41
2.8 Biochemical analyses	42
2.8.1 Lipid peroxidation	42
2.8.2 Na ⁺ /K ⁺ -ATPase (NKA).....	42
2.8.3 Bradford protein assay.....	43

2.9 Exposures	44
2.9.1 Experimental setup	44
2.9.2 Oxygen consumption	44
2.9.3 Ammonia	46
2.10 Ussing chamber	46
2.11 Statistical analyses	47
Chapter 3: The impacts of stress on sodium metabolism and copper accumulation in a freshwater fish	48
3.1 Introduction	49
3.2 Methods	50
3.2.1 Na ⁺ influx	51
3.2.2 Na ⁺ efflux	51
3.2.3 Cu exposure	52
3.2.4 Statistical analyses	53
3.3 Results	53
3.3.1 Na ⁺ influx	53
3.3.2 Na ⁺ efflux	55
3.3.3 Opercular frequency	57
3.3.4 Cu exposure	59
3.4 Discussion	60
3.4.1 Na ⁺ metabolism	60
3.4.2 Cu accumulation	62
3.4.3 Environmental implications	63
Chapter 4: Assessment of trace element accumulation in inanga using an <i>in situ</i> caging study	65
4.1 Introduction	66
4.2 Methods	67
4.2.1 Stream survey	67
4.2.2 Fish survey	69
4.2.3 Caging study	69
4.2.4 ICP-MS	71
4.2.5 Metal speciation	71
4.2.6 Statistical analyses	71
4.3 Results	72
4.3.1 Stream survey	72

4.3.2 Caging study.....	73
4.4 Discussion.....	79
4.4.1 Stream survey	79
4.4.2 Caging study.....	79
Chapter 5: Assessing trace element toxicity in inanga, <i>Galaxias maculatus</i> , using biomarkers after an <i>in situ</i> caging study along the West Coast of New Zealand's South Island.....	83
5.1 Introduction	84
5.2 Methods.....	86
5.2.1 Study sites	86
5.2.2 Fish collection	87
5.2.3 Caged exposures	87
5.2.4 Analytical chemistry and biomarker analyses.....	88
5.2.5 Statistical analyses	88
5.3 Results.....	88
5.3.1 Fish mortality	88
5.3.2 Water chemistry	89
5.3.3 Metal accumulation in fish.....	92
5.3.4 Na ⁺ /K ⁺ ATPase	98
5.3.5 Lipid peroxidation	99
5.4 Discussion.....	101
5.4.1 Whole body trace element accumulation	101
5.4.2 The influence of pH.....	103
5.4.3 NKA.....	104
5.4.4 Regulatory context.....	105
Chapter 6: Evaluating Fe and Zn toxicity in inanga (<i>Galaxias maculatus</i>) through <i>in vivo</i> laboratory exposures.....	107
6.1 Introduction	108
6.2 Methods.....	110
6.2.1 Animal care	110
6.2.2 Experimental setup	110
6.2.3 Physiological endpoints	111
6.2.4 Dissections & digestions	111
6.2.5 Biochemical assays.....	111
6.2.6 Ca ²⁺ influx	112

6.2.7 Na ⁺ influx	112
6.2.8 Statistical analyses	113
6.3 Results	113
6.3.1 Water chemistry	113
6.3.2 Fish metal accumulation	114
6.3.3 Respiration	115
6.3.4 Ammonia	117
6.3.5 Lipid peroxidation	118
6.5.6 Ion influx	119
6.4 Discussion.....	120
6.4.1 Physiological and biochemical toxicity endpoints	121
6.4.2 Whole body accumulation	123
6.4.3 Ion balance	123
6.4.4 Future perspectives	124
Chapter 7: Cutaneous ion transport of sodium and calcium in a scaleless galaxiid; implications for contaminant exposure	125
7.1 Introduction	126
7.2 Methods.....	128
7.2.1 Fish collection and husbandry	128
7.2.2 Partitioning.....	129
7.2.3 Ussing chamber.....	130
7.2.4 Fluorescent dye techniques	131
7.2.5 Statistical analyses	132
7.3 Results.....	133
7.3.1 Partitioning.....	133
7.3.2 Ussing chamber.....	135
7.3.3 Fluorescent dye.....	138
7.4 Discussion.....	139
7.4.1 Cutaneous Na ⁺ transport	140
7.4.2 Cutaneous Ca ²⁺ transport.....	140
7.4.3 The importance of mucus	142
7.4.4 Scaling effects	143
7.4.5 Metal toxicity	144
7.4.6 Mitochondria-rich cells	144

7.4.7 Chromatophores	145
Chapter 8: General discussion	146
8.1 Ion transport in inanga.....	147
8.2 Metal toxicity	148
8.3 Methodological considerations	150
8.4 Environmental perspectives	151
8.5 Regulatory context.....	152
8.6 Future research.....	153
Appendices.....	156
References	163

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Abstract

Inanga (*Galaxias maculatus*) are a culturally and economically important fish species in New Zealand and abroad. However, very little is known about their ability to deal with trace element contamination. As a scaleless fish with the ability to survive in relatively extreme environments, they may not fit toxicity models (such as the biotic ligand model; BLM) based on other fish species. The aim of this study was to determine how this fish responds to elevated trace elements in both the laboratory and field in order to determine the applicability of these toxicity models.

In order to determine the impacts of stress on ion transport and subsequent metal toxicity, inanga were exposed to handling stress and measures of ion uptake were collected. Handling stress was shown to result in increased ventilation rates, resulting in stimulated sodium (Na^+) efflux. A compensatory increase in Na^+ influx was also measured as a result of this stress. Inanga largely recovered from this ionoregulatory stress within 2 hours, with full recovery after 24 hours. This was indicative of a rapid homeostatic response for maintaining ion balance. Enhanced Na^+ uptake in response to this stress resulted in increased copper (Cu) uptake in Cu-contaminated water, suggesting stressed fish will accumulate more Cu (and likely other Na^+ mimics) than an unstressed fish. These results suggest a heightened vulnerability of inanga to this type of contaminant as a result of exercise stress during migrations.

A combination of field and laboratory studies was used in order to measure trace element accumulation in inanga. *In situ* field studies showed changes to aluminum (Al) and iron (Fe) body burdens when inanga were placed in streams of varying trace element concentrations along the West Coast of the South Island. However, other trace elements measured did not alter over the period of exposure (9-10 days). Biochemical biomarker analysis showed no changes in the activity of Na^+/K^+ -ATPase (NKA), but a marker of lipid peroxidation (thiobarbituric acid reactive substances; TBARS) was elevated in one stream. Analysis suggested that stream pH was the major driver of this effect, whether directly or via changes to metal bioavailability. Subsequent laboratory exposures (96 h) of inanga to 1.2, 2.7, 10.8, and 44 $\mu\text{g L}^{-1}$ dissolved Fe and 5.6, 23.3, 60.7, and 128.7 $\mu\text{g L}^{-1}$ dissolved zinc (Zn) showed no difference in whole body trace element accumulation, ammonia excretion, ion influx (Ca^{2+} and Na^+), and TBARS. There were significant differences in oxygen

consumption (MO_2) after Fe exposures, with increases in the 2.7 and 44 $\mu\text{g L}^{-1}$ dissolved Fe exposures. Laboratory exposure results suggest inanga are relatively insensitive to short-term Fe and Zn exposures.

Both *in vivo* (whole body partitioning) and *in vitro* (Ussing chamber) techniques were used to determine the influence of cutaneous ion transport on preventing trace element accumulation. Results suggest inanga use their skin as an additional site of calcium (Ca^{2+}) and Na^+ uptake. This is the first study to confirm these ion transport capabilities in inanga, and revealed that up to 48% of Na^+ uptake may occur across the skin. Pharmacological inhibition of Ca^{2+} uptake was achieved by known Ca^{2+} channel blockers (verapamil and lanthanum). Furthermore Fe and Zn impaired cutaneous Ca^{2+} transport, indicating that ion transport pathways in the skin modulate in response to these metals.

List of tables & figures

Chapter 1

Figure 1.1 Working cell model for mitochondria-rich cells (MRC) in a freshwater fish gill. PVCs are pavement cells, circles are exchangers, ovals with ATP are pumps, and cylinders are channels. Heavy dotted lines are passive paracellular diffusion, light dotted lines are intracellular movement, and the double lined arrow is passive diffusion. CA is carbonic anhydrase (Wood, 2012).

Figure 1.2 Interaction between chemistry and physiological factors in predicting metal toxicology (Wood, 2012).

Figure 1.3 Inanga (*Galaxias maculatus*) – photo credit: Matt Walters

Figure 1.4 Known distribution of inanga in New Zealand (NIWA, 2015).

Chapter 2

Table 2.1 Water chemistry results for aquarium water

Table 2.2 Mean total and dissolved trace element concentrations ($\mu\text{g L}^{-1}$) \pm standard error of mean for aquarium water as determined by ICP-MS. Cd and Co were below detection limits and were therefore excluded.

Figure 2.1 Cylindrical cages constructed for *in situ* caging study (length = 38 cm, radius = 15 cm). Each cage contained 20 fish.

Chapter 3

Figure 3.1 Mean Na^+ influx rates (J_{in} ; $\text{nmol g}^{-1} \text{h}^{-1}$) for individual ($n=6$) inanga (*Galaxias maculatus*) after handling in experimental water (Day 1), aquarium water (Day 1), and after a 24 hour acclimation period in aquarium water (Day 2). Error bars represent one standard error from the mean. Asterisk denotes a significant difference between treatments (Day 1 and Day 2), as determined by Student's t-test ($\alpha = 0.05$).

Figure 3.2 Mean Na^+ efflux rates (J_{out} ; $\mu\text{mol g}^{-1} \text{h}^{-1}$) for individual ($n=7$) inanga (*Galaxias maculatus*) on the same day of handling (Day 1) and 24 hours later (Day 2). Error bars represent one standard error from the mean. Letters indicate significant differences between treatment means as determined by a two-way ANOVA followed by Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

Figure 3.3 Mean Na^+ efflux rates (J_{out} ; $\mu\text{mol g}^{-1} \text{h}^{-1}$) for grouped ($n=3$) inanga (*Galaxias maculatus*) on the same day of handling (Day 1) and 24 hours later (Day 2). Error bars represent one standard error from the mean. Different letters indicate significant differences between treatment means as determined by a two-way ANOVA followed by Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

Figure 3.4 Mean opercular frequency (beats min⁻¹), n=7, directly after handling (Day 1 – Time 0-3) and 24 hours later (Day 2 – Time 24-27). Error bars represent one standard error from the mean. Different letters indicate significant differences between treatment means as determined by a two-way RM ANOVA and Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

Figure 3.5 Linear regression for mean opercular frequency (beats min⁻¹) and mean individual Na⁺ efflux rates (J_{out} ; $\mu\text{mol g}^{-1} \text{h}^{-1}$) ($r^2 = 0.8706$).

Figure 3.6 Total copper body burden in individual fish exposed to 500 $\mu\text{g L}^{-1}$ CuSO₄ for one hour on the same day of handling stress (Day 1, n= 6) and 24 hours later (Day 2, n=6). Error bars represent one standard error from the mean. Significant difference in the mean denoted with asterisks as determined by a Student's t-test ($\alpha = 0.05$).

Chapter 4

Figure 4.1 Location of sampling points for streams surveyed along New Zealand's West Coast. The source stream located in the town of Westport, and caging study streams (3, 6, and 7) denoted with arrows. Numbers correspond to those in Appendix 2.

Table 4.1 Mean water metal concentrations ($\mu\text{g L}^{-1}$) \pm standard error of mean for source (Oro) and caged streams. Cd and Cr had more than 50% of samples below detection limits and were therefore excluded.

Table 4.2 Physicochemical parameters and number of fish collected for metal analysis from source stream (Oro) and three caging study streams (DMC, FN1, and JC).

Figure 4.2 A) Al, B) Cu, and C) Pb (mg kg^{-1}) accumulation in whole versus incomplete fish sample types in caged (DMC, FN1, and JC) and source (Oro) streams for both sample periods combined (96 h and 9 d). Asterisks denote significant differences between whole and incomplete sample types (one-way ANOVA, $p < 0.05$, n values denoted in Table 4.5).

Table 4.3 Mean sediment metal concentrations (mg kg^{-1}) \pm standard error of mean for source (Oro) and caged streams. Asterisk (*) indicate significant differences in trace element concentrations when compared to Oro. Cd had more than 50% of samples below detection limits and was therefore excluded.

Table 4.4 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for combined fish sample types (whole and incomplete fish) (combined 96 hour and 9 day exposure periods). Asterisk (*) indicate significant differences ($p < 0.05$) from the source stream (Oro). Cd, Co, and Cr had more than 50% of samples below detection limits and were therefore excluded.

Table 4.5 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for whole and incomplete fish samples (combined 96 hour and 9 day exposure periods). Asterisk (*) indicate significant differences ($p < 0.05$) between fish accumulation in caged streams *versus*

the source stream (Oro). Cd, Co, and Cr had more than 50% of samples below detection limits and were therefore excluded.

Chapter 5

Figure 5.1 Location of streams along New Zealand's West Coast: source stream denoted in black, caged streams in grey.

Table 5.1 Physiochemical parameters for source (Oro) and caging study streams.

Table 5.2 Mean total and dissolved water trace element concentrations ($\mu\text{g L}^{-1}$) for source (Oro) and caged streams. Cd, Co, and Pb had more than 50% of samples below detection limits and were therefore excluded. ANZECC trigger values ($\mu\text{g L}^{-1}$) for protection of 95% of freshwater biota are included for reference (ANZECC/ARMCANZ, 2000).

Table 5.3 Mean sediment metal concentrations (mg kg^{-1}) for source (Oro) and caged streams; n values were based on separate collection times. Cd had more than 50% of samples below detection limits and was therefore excluded.

Table 5.4 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for 96 hour and 9 day exposure periods. Asterisk (*) indicates significant differences from the source (Oro). Cd, Co, Cr, and Pb had more than 50% of samples below detection limits and were therefore excluded.

Figure 5.2 Fish Al accumulation (mg kg^{-1}) across source and caged streams (n-values in Table 5.1). A single asterisk (*) denotes significant difference from the source, while an asterisk with bar (*—) denotes an overall site difference from the source (two-way ANOVA).

Figure 5.3 Linear correlations between fish Al accumulation (mg kg^{-1}) and A) total water Ni ($\mu\text{g L}^{-1}$), B) dissolved water Ni ($\mu\text{g L}^{-1}$), and C) sediment Ni (mg kg^{-1}) for data collected at both 96 h and 9 d. (Pearson's product-moment correlation, total $p < 0.01$, dissolved $p = 0.03$, sediment $p = 0.03$).

Figure 5.4 Linear correlation between fish Al accumulation (mg kg^{-1}) after both exposure periods and levels of pH in caging streams (Pearson's product-moment correlation, $p < 0.01$).

Figure 5.5 Measures of NKA activity ($\mu\text{mol ADP mg protein}^{-1} \text{ hour}^{-1}$) in the gills at 96 hour and 10 day collection periods for source (Oro) and caging study streams

Table 5.5 N-values for NKA and LPO biochemical assays.

Figure 5.6 Measures of lipid peroxidation in the liver, as measured by MDA activity ($\text{nmol MDA mg protein}^{-1}$), at 96 hour and 10 day collection periods for source (Oro) and caging study streams. Asterisk (*) denotes a significant difference relative to the source stream.

Figure 5.7 Linear correlation between measures of lipid peroxidation in the liver (TBARS) measured by MDA activity (nmol MDA mg protein⁻¹) and levels of pH in caging streams (Pearson's product-moment correlation, $p < 0.01$).

Table 5.6 Results of Pearson's product-moment correlations in relation to TBARS (nmol MDA mg protein⁻¹).

Chapter 6

Table 6.1 Water metal concentrations for exposures (mean \pm standard error of the mean)

Figure 6.1 Whole body accumulation (mg kg⁻¹) for Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L⁻¹ Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

Figure 6.2 Metabolic rates (MO₂, $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$) as determined *via* closed box respiration after Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L⁻¹ Fe which only had 2). Bars sharing letters are not statistically significantly different (one-way ANOVA).

Figure 6.3 Ammonia production (nmol g⁻¹ h⁻¹) measured after Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L⁻¹ Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

Figure 6.4 Levels of oxidative stress as measured by TBARS production (nmol MDA mg protein⁻¹) after Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L⁻¹ Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

Figure 6.5 Ca²⁺ influx (nmol g⁻¹ h⁻¹) in fish after 96 h exposures to A) Fe ($n = 7$) and B) Zn ($n = 8$). There were no significant differences between treatment groups (Student's t-test).

Figure 6.6 Na⁺ influx (nmol g⁻¹ h⁻¹) in fish after 96 h exposures to A) Fe ($n = 8$) and B) Zn ($n = 8$). There were no significant differences between treatment groups (Student's t-test).

Chapter 7

Figure 7.1 Partitioning schematic for separation of head from body. Dotted lines are rubber dental dams.

Figure 7.2 Mean Na⁺ influx ($n = 13$ head, $n = 10$ tail) (nmol g⁻¹ h⁻¹) \pm standard error of the mean in the head and tail regions of inanga skin after *in vivo* partitioning.

Figure 7.3 Mean Ca²⁺ influx ($n = 5$ head, $n = 5$ tail) (nmol g⁻¹ h⁻¹) \pm standard error of the mean in the head and tail regions of inanga skin after *in vivo* partitioning. Asterisk (*) denotes a significant difference (Student's t-test).

Figure 7.4 Non-linear least squares regression for Na^+ influx ($\text{nmol g}^{-1} \text{h}^{-1}$) in the head portion of inanga ($n = 23$) as it relates to total fish mass (g) after *in vivo* partitioning.

Figure 7.5 Mean Ca^{2+} influx (J_{ms} , $\text{nmol cm}^{-2} \text{h}^{-1}$) \pm standard error of the mean in the head ($n = 18$) and tail ($n = 17$) portion of inanga skin after *in vitro* Ussing chamber experiments.

Figure 7.6 Mean Ca^{2+} influx (J_{ms} , $\text{nmol cm}^{-2} \text{h}^{-1}$) \pm standard error of the mean in the head portion of inanga skin as controls ($n = 2$), and after treatments ($n = 4$ each) of Fe ($350 \mu\text{M}$), Zn ($100 \mu\text{M}$), La ($1 \mu\text{M}$), and verapamil ($25 \mu\text{M}$) using an *in vitro* Ussing chamber technique. Bars sharing letters do not differ significantly in Ca^{2+} influx rates (one-way ANOVA).

Figure 7.7 Mean Ca^{2+} influx (J_{ms} , $\text{nmol cm}^{-2} \text{h}^{-1}$, $n = 17$) and Ca^{2+} efflux (J_{sm} , $\text{nmol cm}^{-2} \text{h}^{-1}$, $n = 18$) \pm standard error of the mean in combined head and tail portions of inanga skin. Asterisk denotes a significant difference between influx rates as determined by a one-way ANOVA.

Figure 7.8 Image of the skin of inanga posterior to the head for control fish (no dye) with reflective star-shaped cells. The image was captured using a confocal laser-scanning microscope with a 10x lens and an excitation wavelength of 530nm.

Figure 7.9 Image of the skin of inanga posterior to the head after being exposed to Mitotracker green dye (1 mM) for 2 hours (green cells are putative MRC). The image was captured using a confocal laser-scanning microscope with a 10x lens and an excitation wavelength of 530nm.

Appendices

Appendix 1. Physiochemical water parameters and fish caught for the 34 streams surveyed along the West Coast of New Zealand's South Island in February 2013.

Appendix 2. Total water trace element concentrations ($\mu\text{g L}^{-1}$) for the 34 streams surveyed ($n = 1$ each) along the West Coast of New Zealand's South Island in February 2013. Cd and Pb had more than 50% of samples below detection limits and were therefore excluded.

Appendix 3. Total sediment trace element concentrations (mg kg^{-1}) \pm standard error of mean for the 34 streams surveyed ($n = 2$ each) along the West Coast of New Zealand's South Island in February 2013. Cd had more than 50% of samples below detection limits and was therefore excluded.

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BLM	Biotic ligand model
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CAT	Catalase
cpm	Counts per minute
CRM	Certified reference materials
CTR1	Copper transporter 1
DMT1	Divalent metal transporter
DO	Dissolved oxygen
ECaC	Epithelial calcium channel
ENaC	Epithelial sodium channel
GI	Gastrointestinal
GR	Glutathione reductase
GST	Glutathione-S-transferase
IB	Imidazole buffer
J_{ms} or J_{in}	Ion influx
J_{sm} or J_{out}	Ion efflux
LC ₅₀	Median lethal concentration
LOEC	Lowest observed effect concentrations
LPO	Lipid peroxidation
MDA	Malondialdehyde
MO ₂	Oxygen consumption
MRC	Mitochondria-rich cells
MS-222	3-aminobenzoic acid ethylester
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NCX	Na ⁺ /Ca ²⁺ exchanger
NHE	Na ⁺ /H ⁺ exchanger
NKA	Na ⁺ /K ⁺ -ATPase
NOM	Natural organic matter
Oro	Orowaiti Lagoon
PBS	Phosphate buffered saline
PO ₂	Partial pressure of oxygen
PVC	Pavement cell
Rh	Rhesus proteins
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEP	Transepithelial potential
V-ATPase	Proton pump
ZTL	Zn transporter

Chapter 1: Introduction

1.1 Background

Fish are constantly faced with the task of maintaining internal ion concentrations in an osmotically challenging environment (Evans et al., 2005). In addition, anthropogenic sources of contamination can, among other effects, further exacerbate these challenges to osmoregulation (Wood, 2012). The addition of trace elements to streams is particularly relevant to the freshwater biota in New Zealand (Greig et al., 2010). Trace elements are defined as chemical elements present in minute amounts. These elements are naturally present at low levels in all freshwater streams, with some necessary as nutritional requirements (Watanabe et al., 1997). However, at high levels, trace elements can cause toxic effects to aquatic biota (Wood, 2012).

Anthropogenic inputs are often the cause of increased trace element concentrations in freshwater streams (Wood, 2012). In New Zealand, anthropogenic sources of trace elements include industrial discharge, urban or agricultural runoff, and mining (Greig et al., 2010; Williamson, 1985). The toxic effects of these elements will depend on the concentration, mechanisms of toxicity, and water chemistry (Di Toro et al., 2001). As such, determining the specific mechanisms behind trace element toxicity is an important step in preventing toxicological consequences. Physiology has also been shown to affect toxicity, with different species displaying different means of absorbing and handling trace elements, thus contributing to different trace element sensitivities (Besser et al., 2007). Determining how these trace elements affect New Zealand freshwater fish is an important step in protecting these native fauna.

1.2 Osmoregulation in freshwater fish

Freshwater fish maintain internal ion concentrations greater than those of their surroundings and are therefore faced with ion loss and water gain (Evans et al., 2005). In order to maintain this concentration gradient, freshwater fish must constantly take up ions from the water while simultaneously preventing ion loss. Historically, the two sites of ion uptake are the gills and intestine, with the gills being the main site of exchange (Evans, 2011). At the gills, ion transport is intricately associated with other vital functions such as nitrogenous waste excretion and acid-base balance (Evans et al., 2005). The kidney is also an

important site for osmoregulation in fish, but takes a secondary role relative to the gill (Evans, 2010). Within the gills, mitochondria-rich cells (MRC) are the primary site for ion transport. These cells contain large amounts of mitochondria and various ion transporters (Fig. 1.1); all of which are necessary for ion exchange (Evans, 2011). Two to three different types of MRC have been identified in freshwater fish, each of which has a distinct morphology, assortment of transporters, and response to different ionic conditions (Hwang et al., 2011). Pavement cells (PVC) are also thought to participate in ion transport, albeit to a lesser degree than MRC (Hwang et al., 2011).

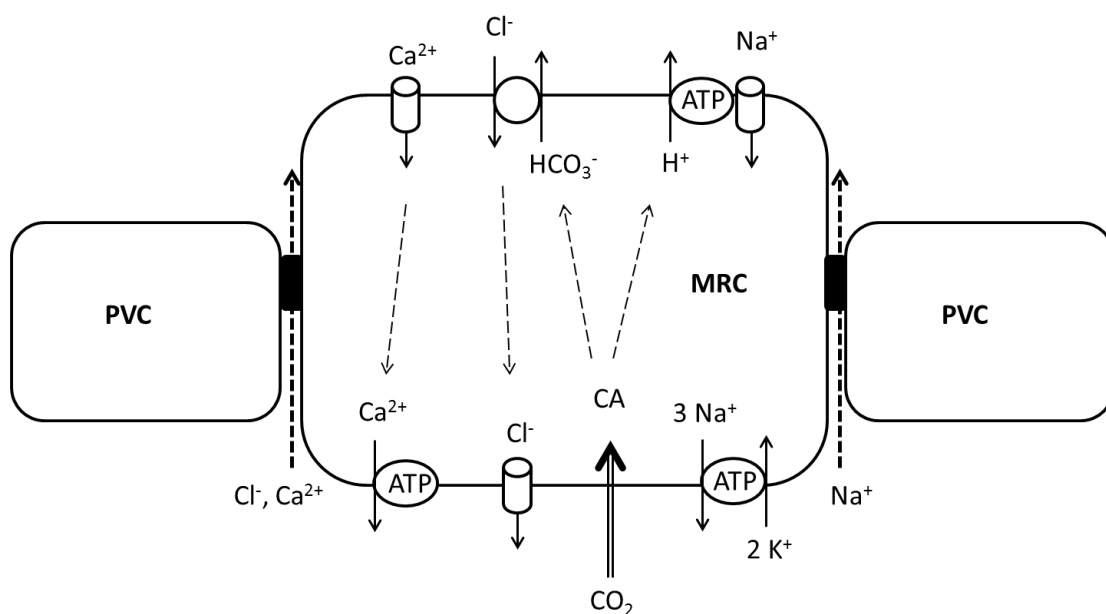


Figure 1.1 Working cell model for mitochondria-rich cells (MRC) in a freshwater fish gill. PVCs are pavement cells, circles are exchangers, ovals with ATP are pumps, and cylinders are channels. Heavy dotted lines are passive paracellular diffusion, light dotted lines are intracellular movement, and the double lined arrow is passive diffusion. CA is carbonic anhydrase (Wood, 2012).

Two of the most studied and best understood ion transport pathways are those for sodium (Na^+) and calcium (Ca^{2+}). Na^+ is one of the most important ions for maintaining the resting membrane potential, and therefore the ability of the cell to transport other ions (Evans, 2008; Handy et al., 2002). Ca^{2+} , on the other hand, is necessary for muscle contraction, neural and intercellular communication, intracellular signaling, regulation of enzymatic reactions, and reproduction (Guerreiro and Fuentes, 2007). Na^+ influx is thought

to occur through one of three mechanisms. It can be coupled with hydrogen ion extrusion via a Na^+ -hydrogen exchanger (NHE) or an epithelial Na^+ channel (ENaC) which is linked to a proton pump (V-ATPase) (Evans, 2011). Recently, the coupling of Na^+ uptake to ammonia excretion *via* Rhesus proteins (Rh) on the apical surface of MRC has also been proposed to significantly contribute to Na^+ uptake (Wright and Wood, 2009). After entry into the cell, Na^+ then enters the blood via the basolateral Na^+/K^+ -ATPase (NKA) (Fig. 1.1). Ca^{2+} influx is driven by a concentration gradient created by low cellular Ca^{2+} concentrations. Ca^{2+} enters the cell *via* facilitated diffusion through apical Ca^{2+} channels (ECaC or equivalent) (Flik and Verbost, 1993; Hwang and Lee, 2007). After Ca^{2+} enters the cell, it is then transported to the blood either through a basal Ca^{2+} -ATPase (Fig. 1.1), or exchanged for Na^+ *via* a NCX transporter (Na^+ and Ca^{2+} exchanger) which relies on the Na^+ gradient produced by NKA (Dymowska et al., 2012; Hwang and Lee, 2007). Both of these ions require a functioning NKA in order for uptake to occur, underlying the importance of this pump to osmotic balance. While the gill is of primary importance for ion regulation in most freshwater fish, it may have a relatively lesser role in some species that are able to use the skin as a transport surface (see Section 1.7 below).

1.3 Trace element toxicity

1.3.1 Sources of trace elements

The term trace element is used throughout this thesis in regards to both metals and metalloids, while the term metal is strictly used in regard to elements categorized as metals. There are both anthropogenic and natural sources of trace element contaminants in the aquatic environment. Natural sources primarily include wind-borne particles, volcanism, weathering of mineral-rich substrates, and forest fires; while anthropogenic inputs vary from concentrated industrial point sources to diffuse sources associated with general human activities (Nriagu, 1989; Wood, 2012). In New Zealand, acid mine drainage and urban and agricultural runoff appear to be of the greatest significance (Greig et al., 2010; McCauley et al., 2006; Paul and Meyer, 2001; Wang et al., 2004; Winterbourn, 1998). Sites affected by acid mine drainage can have exceedingly high concentrations of both aluminum

(Al) and iron (Fe) (McCauley et al., 2006; Winterbourn, 1998). Resulting acidity can also lead to the leaching of Fe from ambient rock formations, increasing dissolved, and therefore bioavailable, species of the metal (McCauley et al., 2006). Fe contamination is therefore most prevalent in the coal mining regions on the West Coast where it is associated with acid mine drainage (Greig et al., 2010).

Metal contamination resulting from urban runoff includes lead (Pb), zinc (Zn), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), cadmium (Cd), and mercury (Hg). In New Zealand, both Cu and Zn are a priority in terms of monitoring levels for freshwater biota (ANZECC/ARMCANZ, 2000; Paul and Meyer, 2001). In particular, urban stormwater drainage is thought to be a significant contributor of Zn and Cu contamination. Runoff from cars, copper-paneled roofing, and galvanized steel can all lead to elevated Cu and Zn concentrations (Brown and Peake, 2006; O'Sullivan et al., 2012). As stormwater can drain into spawning and migratory waters for local fish species, this source can have an important impact on aquatic biota (Williamson, 1985).

1.3.2 Bioavailability

There is a large body of literature attempting to predict the effects of trace element contaminants on aquatic life (Besser et al., 2007; Cooper et al., 2009; Greig et al., 2010; Hogsden and Harding, 2012; Norwood et al., 2003; Reynders et al., 2008). The two main sites of absorption in fish are across the gills (waterborne) and intestine (dietary) (Clearwater et al., 2002). Which pathway dominates is likely related to the speciation of the metal and where it comes into contact with epithelia.

One assumption is that waterborne trace element toxicity can largely be explained by the speciation of the trace element. This is because the chemical speciation determines its bioavailability (the degree to which the metal can be taken up by an organism) to the gill and hence its toxicity through accumulation. Speciation, however, depends on a large number of variables, including salinity, pH, water hardness, alkalinity, and natural organic matter (NOM) (Fig. 1.2; Di Toro et al., 2001; Dutton and Fisher, 2011; Niyogi and Wood, 2004). While predicting speciation as a result of these variables is relatively straight forward, determining how speciation affects bioavailability and subsequent toxicity is quite difficult. In waterborne exposures, the free ionic form of the trace element is considered the most

bioavailable, as some are able to mimic other common ions (i.e. Na^+ and Ca^{2+} , as mentioned below) to gain entry into the gill (Wood, 2012). Essential waterborne trace elements also need to be in their free ionic form in order to be absorbed through apical membrane transporters that are specifically present to facilitate their uptake into the animal and thus meet nutritional needs. These transporters include Cu transporter 1 (CTR1), divalent metal transporter (DMT1), and Zn transporter (ZTL) (Bury et al., 2003).

Water chemistry factors each have varying effects on trace element toxicity. Factors such as NOM, water hardness, pH, salinity, and alkalinity can alter the chemical speciation of trace elements (Di Toro et al., 2001; Wood, 2012). This in turn has a significant impact on bioaccumulation and toxicity in fish. Consequently, knowledge of water chemistry is critical to understanding the sensitivity of fish to metals. Salinity, for example, is thought to decrease metal bioavailability and therefore toxicity (Dutton and Fisher, 2011; Lee et al., 2010). The high ion content in saline water is thought to compete with free, ionic metals for transmembrane ion transporters of the gill (Bury et al., 2003). As different metals use different ion transporters, the ameliorative effect of salinity on toxicity varies for each metal (Dutton and Fisher, 2011; Lee et al., 2010). Similarly, water hardness was shown to protect fathead minnows against Ni toxicity (Pyle et al., 2002), presumably due to competition between Ni and Ca^{2+} /magnesium (Mg^{2+}) for uptake sites (Deleebeeck et al., 2007). The effect of pH on bioavailability varies by metal and ionic composition of the water (Schubauer-Berigan et al., 1993). For example, low pH waters can either decrease the absorption of metal ions, due to competition with protons (Heijerick et al., 2002), or increase absorption as a result of increased free metal ions, the form generally considered most bioavailable (De Schamphelaere and Janssen, 2002; Meador, 1991). NOM is a natural component of streams that may act as a complexing agent for metals. For example, increased levels of NOM were shown to protect rainbow trout from Cu, Cd, and Zn toxicity (Kamunde and MacPhail, 2011). However, the ability to bind metals depends on specific NOM qualities; with aromatic humic substances being the dominant metal chelator (Baken et al., 2011). Although most bound metals are considered unavailable for uptake, those bound to amino acids may still be bioavailable (Glover and Hogstrand, 2002a), making toxicity predictions based on dissolved concentrations insufficient. The interaction between different metals in a mixture is also an important factor, leading to increased or decreased

metal uptake, depending on the nature of the mixture and whether these metals share similar uptake pathways (Cooper et al., 2009).

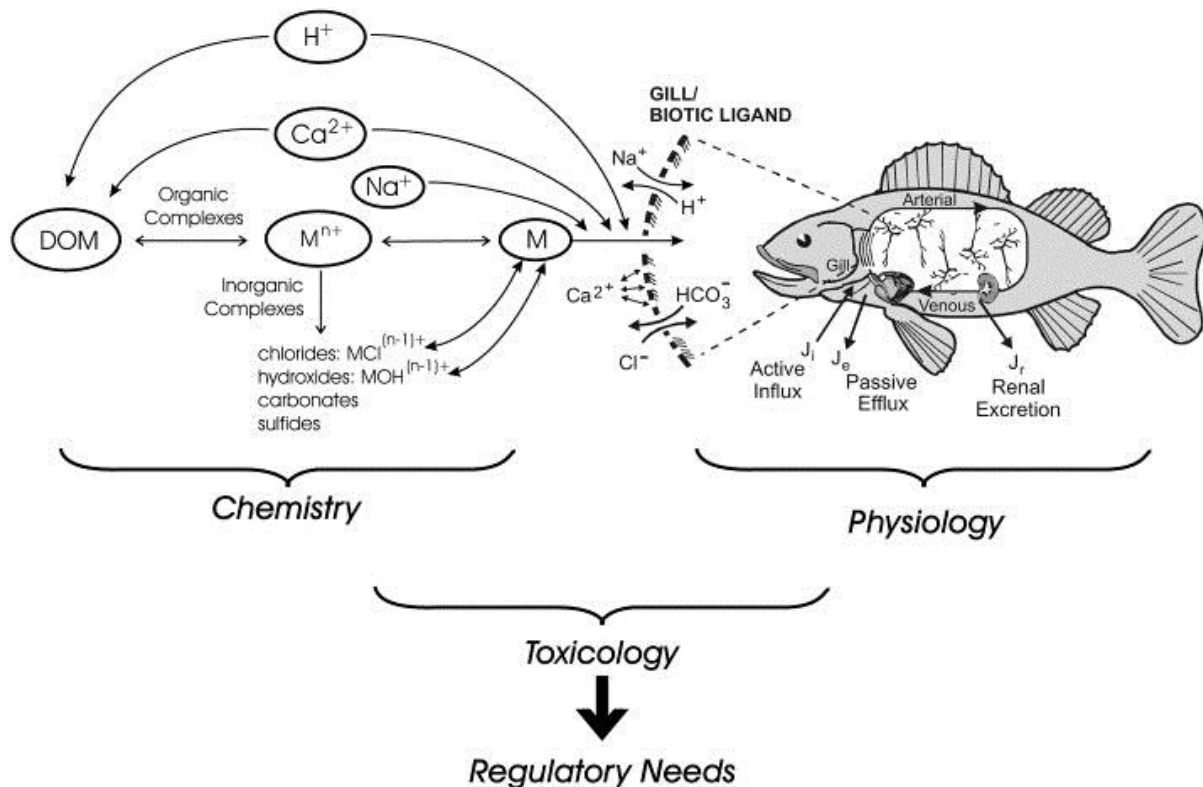


Figure 1.2 Interaction between chemistry and physiological factors in predicting metal toxicology (Wood, 2012).

1.3.3 Trace element uptake

The physiological mechanisms by which trace elements exhibit their toxic effects, and the amount needed to exhibit these effects, vary with each trace element (Paquin et al., 2002; Wood, 2012). One of the primary mechanisms of toxicity relates to the specific pathway by which each element is absorbed. Metal ions can utilize pathways used by aquatic animals for ion regulation, and in doing so, disrupt this transport. For freshwater fish, the toxic effects of trace elements are generally thought to impact the gill, as it is the

main site of ion regulation (see Section 1.2). Commonly divalent metals such as cobalt (Co), Cd, Pb, and Zn compete with Ca^{2+} uptake, leading to hypocalcemia (Spry and Wood, 1985). Similarly, monovalent elements will compete with Na^+ uptake, resulting in hyponatremia (Grosell and Wood, 2002). In addition to the disruption of ion homeostasis, metals such as Al and Fe can accumulate on the gills impairing respiration and ion transport through gill clogging and damage (Dalzell and MacFarlane, 1999; Gensemer and Playle, 1999). It is important to note that some fish species are known to physiologically alter their ion transport properties in order to minimize metal accumulation and toxicity. For example, salmon are capable of recovering whole body Na^+ concentrations after chronic Cu exposures by reducing Na^+ efflux (Laurén and McDonald, 1987b). This ability, however, is thought to vary between species due to physiological differences (Hogstrand et al., 1995).

The uptake of trace elements in the gastrointestinal (GI) tract will directly depend on those present in the diet, as freshwater fish do not drink. To a certain extent, metal absorption can be controlled by the organism; particularly essential micronutrients like Cu, Fe, and Zn that have their own homeostatic regulatory mechanisms (Bury et al., 2003; Clearwater et al., 2002). The majority of uptake for nutritionally required metals occurs in the GI tract (Bury et al., 2003). However, dietary requirements for each vary. For example, the dietary requirements of fish for Cu range between 1-5 mg kg^{-1} dry mass, Fe between 30-170 mg kg^{-1} dry mass, and Zn between 15-40 mg kg^{-1} dry mass (Watanabe et al., 1997). As the gill is the main site of ion regulation and there are typically lower concentrations of free, ionic forms available in the gut, dietary metal exposures are often less toxic than waterborne exposures (Ojo and Wood, 2007). This has been seen with Zn uptake in yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*) (Glover and Hogstrand, 2002b; Niyogi et al., 2007). However, in excess, these metals have been shown to inhibit growth rates, reduce immune function, and even threaten survival (Clearwater et al., 2002).

1.3.4 Toxic effects of Fe, Zn, and Cu

One of the major trace elements examined in this study is Fe, as it was found at elevated concentrations among streams surveyed (Chapter 4). Fe is an important component in heme groups necessary for oxygen transport and as cytochromes in the

electron transport chain (Bury and Grosell, 2003a). The toxic effects of Fe on freshwater fish gills make waterborne Fe exposures more toxic than dietary exposures (Bury and Grosell, 2003b; Bury et al., 2001; Dalzell and MacFarlane, 1999). As such, determining the chemical species of Fe is an important step in predicting toxicity, as the ferrous state (Fe^{2+}) is considered more bioavailable than the ferric state (Fe^{3+}) (Bury et al., 2003). In aerobic environments, Fe is more often found as ferric (hydro) oxides; however, with decreasing pH, concentrations of ferrous iron increase (Bury et al., 2012). The ability of Fe^{2+} to produce hydroxyl radicals via the Fenton reaction (Equation 1.1) is one potential mode of toxicity (Crichton et al., 2002). The Fenton reaction is the Fe-dependent decomposition of hydrogen peroxide, resulting in the production of a hydroxide anion and one highly reactive hydroxyl radical. The accumulation of these reactive oxygen species (ROS) can subsequently lead to oxidative stress responses within the cell (Crichton et al., 2002).

Equation 1.1: The Fenton reaction:



Another trace element on which this research is focused is Zn, as its mechanism of toxicity is relatively well understood (Hogstrand, 2012). Zn is required for proteins involved in growth, reproduction, and immune function (Bury et al., 2003). While most nutritionally required Zn is taken up *via* the GI tract, waterborne Zn is more toxic due to constant exposure to the affected ligand (Clearwater et al., 2002). As Zn competes with Ca^{2+} uptake, increased Ca^{2+} concentrations have been shown to result in decreased Zn toxicity (De Schamphelaere and Janssen, 2004). Acute Zn exposures have also been shown to result in increased ammonia excretion, which can then lead to metabolic acidosis, as acidic equivalents are taken up when ammonia is excreted through the gill (Spry and Wood, 1985). The lowest observed effect concentrations (LOEC) for survival as a result of chronic Zn exposures range from $27 \mu\text{g L}^{-1}$ to 2.3 mg L^{-1} (De Schamphelaere and Janssen, 2004).

The mechanisms of Cu toxicity are also relatively well understood. Cu is a nutritionally required metal that serves as an important co-factor for many proteins including cytochrome c, and proteins used in cellular respiration (Bury et al., 2003). Cu is more common as a divalent ion in freshwater; however, it is thought to be reduced to the

monovalent ion, likely through an apical reductase on the gill, before being able to traverse Na^+ pathways (Bury et al., 2003). This Na^+ competition can then lead to reduced Na^+ uptake, eventually leading to hyponatremia; which if severe enough, can be lethal (Grosell and Wood, 2002). Cu uptake can be well regulated in both the gills and GI tract, however, the gill is more often the site of toxicity as it is the dominant locus of Na^+ ion regulation (Grosell and Wood, 2002). For waterborne Cu exposures, Cu concentrations at which 50% of fish exhibit mortality (96 h LC_{50}) was shown to range from 91-153 $\mu\text{g L}^{-1}$, depending on water hardness, in rainbow trout (Taylor et al., 2000).

Species-specific physiology can also influence the toxicity of trace elements, with some organisms being more susceptible than others. For example, fish with high Na^+ turnover rates are likely to be more susceptible to Na^+ antagonists (i.e. Cu and silver (Ag)) (Grosell et al., 2002). These organisms have higher Na^+ requirements; therefore inhibition of uptake is more harmful than for organisms with lower Na^+ requirements (Grosell et al., 2007). As such, size also plays an important role in influencing Ag and Cu toxicity, as Na^+ turnover rates are higher in smaller animals (Grosell et al., 2002).

Fish can also mitigate the toxic effects of trace element exposure through physiological defenses. For example, the production of anti-oxidant enzymes (catalase (CAT), superoxide dismutase (SOD), and glutathione-related enzymes (glutathione-S-transferase (GST), glutathione reductase (GR), etc.)) can neutralize ROS (see Section 1.5) before it damages the cell (Bagnyukova et al., 2006). The synthesis of proteins capable of binding and storing metals (i.e. metallothionein) can also function to sequester and detoxify metals at low concentrations. This sequestration, however, is limited in terms of exposure length and concentration (Chowdhury et al., 2005; Kraemer et al., 2005a). Excretion and intracellular partitioning of trace elements in specific tissues can also help decrease toxic impacts and increase the tolerance of fish in contaminated environments (Goto and Wallace, 2010). Fish have also been shown to acclimate to chronic exposures of certain trace elements. For example, rainbow trout were shown to acclimate to Zn-contaminated waters by adjusting uptake rates, albeit at an energetic cost (Hogstrand et al., 1995). McGeer et al. (2000a,b) also showed acclimation came at a metabolic cost through the active regulation of tissue burdens.

1.4 Biotic Ligand Model

Historically, the primary factor determining environmental policy regarding trace elements in aquatic systems has been total water trace element concentrations (Niyogi and Wood, 2004). However, toxicity does not always link directly to total water trace element concentrations, and other factors need to be taken into account. The biotic ligand model (BLM) approach has been adopted as a paradigm to predict trace element concentrations that lead to toxicity. This approach combines biogeochemical modelling (to calculate metal speciation) and trace element uptake (specifically that at the toxicologically-sensitive site, or “biotic ligand”) to predict bioavailability and resulting toxicity of each metal to aquatic organisms (Niyogi and Wood, 2004; Paquin et al., 2002). There is a BLM for a few ecologically relevant metals (Cu, Ag, Zn, and Ni), each of which attempts to account for the effects of water quality parameters on speciation and bioavailability (Niyogi and Wood, 2004). Through this prediction, the BLM provides an ecologically-relevant framework from which environmental policy can be formed.

New Zealand currently follows the Australian and New Zealand Environment and Conservation Council’s (ANZECC) guidelines in determining environmental policy regarding metal contaminants. The BLM is mentioned in these guidelines, but not directly advocated (ANZECC/ARMCANZ, 2000), whereas the newly formed Environmental Protection Authority lists the BLM as a valid method for the ‘classification of metals and metal compounds’ (NZEPA, 2012). Consequently, regional councils in charge of enforcing these policies are left to determine whether or not to incorporate the model in regulations. In comparison, the BLM is used in federal regulatory guidelines in the United States regarding acceptable levels of Cu contaminants in the aquatic environment (U.S. EPA, 2003). Determining whether the BLM approach accurately predicts toxicity in inanga could lead to policy clarifications regarding its use.

One key assumption of the BLM is that the mechanisms of metal absorption are the same for all fish; however, this may be an oversimplification. The biotic ligand for fish, according to the BLM principle, is the gill (Fig. 1.2). As such the BLM does not account for the importance of metal uptake and toxicity in other tissues. Additionally, various physiological factors such as ventilation rate, gill surface area, and diet that are not included in the BLM may also affect the rate at which metals accumulate in freshwater fish

(Clearwater et al., 2002; Grosell et al., 2002). Toxicity has also been shown to change with acclimation. For example, Ca^{2+} transporting capacity was shown to recover after acclimation to Zn-contaminated water in rainbow trout (Hogstrand et al., 1995). Such an effect distorts the relationship between gill metal accumulation and toxic effect. These alterations to trace element toxicity have the potential to make predictions based on the BLM imprecise, particularly for fish with unusual physiological characteristics.

1.5 Biomarkers

An ecotoxicological biomarker has been defined as: “a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)” (Depledge, 1993). A variety of biomarkers have been used in toxicity studies aiming to identify a wide range of biological consequences (Lushchak, 2012).

Measures of oxidative stress have commonly been used as biomarkers in assessing the toxicity of metals to freshwater fish (Lushchak, 2012; van der Oost et al., 2003). Metals typically cause oxidative stress through one of two mechanisms, depending on their valence. Typically, ions with a fixed valence will interfere with metabolic pathways *via* competition with Ca^{2+} and Zn^{2+} , enhancing the production of free radicals, or through antioxidant enzyme inhibition (Lushchak, 2012). Those with a variable valence often enhance ROS production; typically through the Fenton reaction (Equation 1.1) (Lushchak, 2012). These ROS can then lead to DNA, lipid, and protein damage; however, antioxidant enzymes (CAT, SOD, GST) can be used to neutralize ROS before such damage occurs (van der Oost et al., 2003). Fish naturally have high amounts of polyunsaturated lipids that when subject to oxidative stress produce thiobarbituric acid reactive substances (TBARS) (Pedrajas et al., 1995). By measuring TBARS after metal exposure, the amount of oxidative stress due to lipid peroxidation can be estimated (Pedrajas et al., 1995). TBARS have been shown to increase in response to metal exposure for goldfish after Fe exposures (Bagnyukova et al., 2006), catfish after Cu exposures (Hoyle et al., 2007), and killifish after Zn exposure (Loro et al., 2012).

As NKA is responsible for maintaining MRC ion gradients (Section 1.2), the activity of NKA has been used as a biomarker of disturbances in ion transport after metal exposures (Hoyle et al., 2007; Lappivaara and Marttinen, 2005; Laurén and McDonald, 1987a). Metals such as Cd, Cu, Fe, and Zn have all been shown to reduce NKA activity in the gills of freshwater fish (Fernandes et al., 2013; Li et al., 2012; Wu et al., 2008). Specifically, Cu has been shown to bind to the -SH groups of NKA causing a change in the protein shape, thereby resulting in functional changes (Kone et al., 1990). Ag is thought to bind and displace Mg^{2+} from the active site, preventing phosphorylation and impeding function (Ferguson et al., 1996). It does appear that alterations in the synthesis of NKA and/or alterations in the number of MRC in the gills can recover NKA activity as fish acclimate to metal exposures (Wu et al., 2008).

1.6 Approaches to studying trace element toxicity

Investigating the toxicity of trace elements to freshwater fish through the use of laboratory studies has been ongoing for decades (Paquin et al., 2002). Both acute and chronic exposures have been used; each providing important information about the physiological effects of metal exposures. While acute exposures serve to better identify the mechanistic causes of toxicity, chronic exposures provide a more environmentally realistic exposure criterion (Cooper et al., 2009; Dalzell and MacFarlane, 1999). Although it is rare that a fish experiences high doses of contaminants for short periods of time; acute studies are necessary for understanding toxic mechanisms and therefore are aligned with the development of effective regulations, such as the BLM. It is far more likely that contaminants slowly accumulate and linger in an ecosystem, but toxic effects resulting from such chronic exposures are often less prominent (Cooper et al., 2009). As there is limited research on the effects of trace elements on inanga, acute exposures are necessary to discern the mechanistic responses to metal exposures.

Although the bioavailability and toxicity of metals in the aquatic environment has been subject to a great deal of research, results are often inconsistent, especially when comparing field and laboratory research. Most laboratory inconsistencies result from fish being exposed to concentrations and/or conditions in which pharmacological rather than physiological responses are recorded, and which therefore may not necessarily reflect the

actions of the toxicants in natural settings (Kraemer et al., 2005b). While this type of exposure is important for determining mechanisms of toxicity, it does not provide information regarding long term sub-lethal effects. As such, acute toxicity results can only guide environmental regulations in terms of short term exposures. In addition, metals are absorbed, accumulate, and affect each species differently. Most studies agree that contaminant concentration and duration of exposure confer different results; with acute exposure often being at higher concentrations with more exaggerated effects than chronic exposure (Clearwater et al., 2002). It is important to validate these laboratory results in natural settings in order for results to be directly relevant to species conservation. One such experimental approach is the use of caging studies. These studies bridge the gap between laboratory and field studies by mimicking parameters measured in a controlled environment in a more natural setting. Exposing fish from a singular source site to a range of contaminated sites and measuring resulting accumulation allows for a comparison to laboratory results. This can help better identify whether important natural variables unaccounted for in the laboratory setting may be involved in trace element accumulation (Kraemer et al., 2005a, b; Reynders et al., 2008).

1.7 Inanga

Inanga (*Galaxias maculatus*) (Fig. 1.3) is a widely distributed freshwater fish in coastal streams in Australia, New Zealand, South America, Lord Howe Island, the Chatham Islands, and the Falkland Islands (McDowall, 1989; Waters and Burridge, 1999). The widespread distribution of galaxiids is thought to have occurred through multiple Gondwanan vicariations followed by subsequent oceanic dispersals (Burridge et al., 2012). The family Galaxiidae falls under the superorder Protacanthopterygii which includes smelts, salmon, and pike. Inanga are distributed across most coastal areas in New Zealand; however, they are not found more than 10 km inland (Fig. 1.4).

Inanga are an amphidromous species; meaning fish hatch in freshwater streams before drifting to the ocean to develop. Larvae are thought to spend between 3-6 months in the ocean before migrating back to freshwater streams to spawn (McDowall et al., 1994). After spawning, eggs are deposited into riparian vegetation during spring tides. Eggs then hatch and drift to sea after being re-immersed on the next spring tide (Richardson and

Taylor, 2002). In New Zealand, inanga are both a culturally and economically important species. They are the major species in the local 'whitebait' fishery which catches juveniles as they migrate back to freshwater streams in the spring. Whitebait catch can earn between \$28-83 U.S. dollars kg⁻¹ in New Zealand and abroad (Mardones et al., 2008). Inanga are also culturally important, both as a traditional cuisine and as a traditional catch for indigenous Maori populations (McDowall, 1996). Over the past century, whitebait catches have declined as a result of changes in land use, anthropogenic contamination of habitats, and the introduction of invasive salmonids (McDowall, 1984, 2006; Rowe et al., 1999). While concerns have been raised, there is still little in terms of regulatory protection, and the fishery remains highly exploited (McDowall, 2006).

Inanga are capable of tolerating extreme environmental conditions. They have been shown to effectively osmoregulate across a range of salinities (Chessman and Williams, 1975; Urbina et al., 2013), tolerate acidic waters (Glover et al., 2012), and use emersion to deal with hypoxic waters (Urbina et al., 2011). Inanga have also been shown to be insensitive to relatively high levels of ammonia (Richardson, 1997). However, little is known regarding inanga tolerance to metal exposure. One study conducted on inanga eggs and early life-stages showed reduced hatching success and a weaker phototactic response following exposure to Cu, Zn, and Pb mixtures (Barbee et al., 2014). It is, however, predicted that they will be relatively sensitive to metal toxicants with effects on Na⁺-transport pathways. As small fish (between 0.1-4 g), inanga will have a high surface area to volume ratio, a trait that makes them susceptible to these toxicants (Grosell et al., 2002). Determining how trace elements affect ion transport and whether or not cutaneous transport (see below) can help compensate for these toxic effects are important components in understanding how inanga cope with trace element exposures.



Figure 1.3 Inanga (*Galaxias maculatus*) – photo credit: Matt Walters

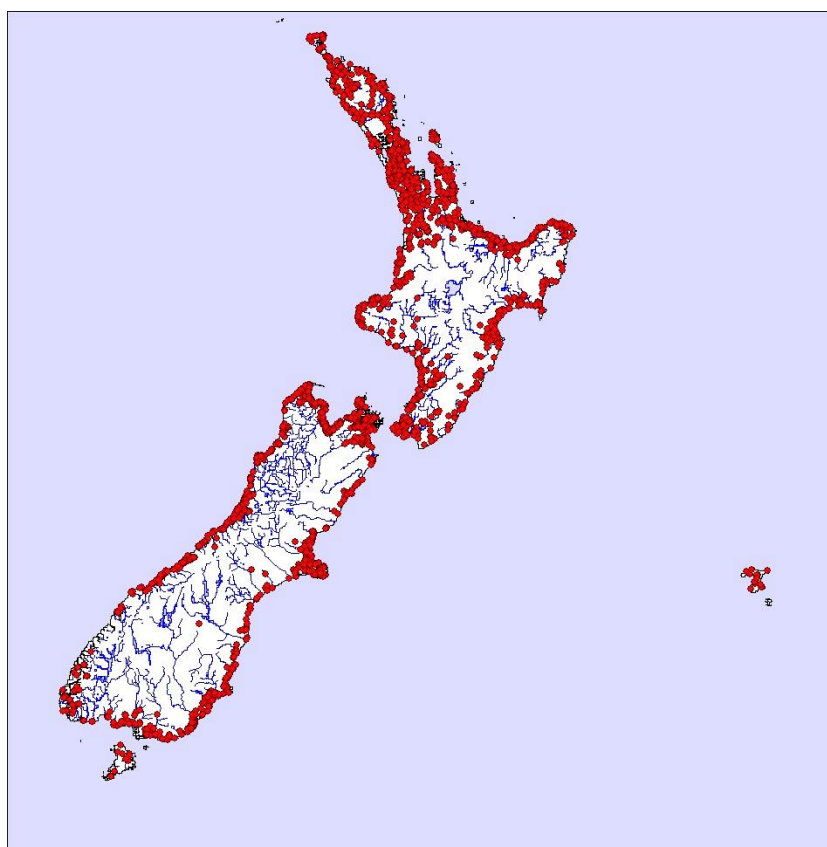


Figure 1.4 Known distribution of inanga in New Zealand (NIWA, 2015).

One of the reasons inanga are a good model for studying ion transport is they lack scales (McDowall, 1989). Scales are thought to be an important factor in minimizing the exchange of ions between the animal and the environment (Glover et al., 2013). Without this barrier, ion transport can more easily occur, making the skin of scaleless fish more likely to participate in ion exchange. However, this increased ion transport may also lead to higher rates of diffusive loss, exacerbating ionoregulatory stress. Inanga are capable of inhabiting highly saline waters (David and Closs, 2003; McDowall, 2006), suggesting they have particularly effective ion regulatory mechanisms. Inanga have also been shown to switch NKA isoforms in skin epithelium in response to salinity changes (Urbina et al., 2013). This implies a change in transport properties, thereby indicating a possible role for the skin in ion homeostasis. While for most fish, ion transport occurs predominately through the gills, those species with well-vascularized skin such as gobies (*Gillichthys mirabilis*), mudskippers (*Periophthalmus modestus*), and blennies (*Blennius pholis*) may use skin to achieve ion balance (Evans and Claiborne, 2006). For example, chloride has been shown to be transported across the skin in multiple fish species (Degnan et al., 1977; Karnaky et al., 1977; Nonnotte et al., 1979). If the skin of inanga proves to play an important role in ion transport, then it may act as an additional site for trace element toxicity, or it might act as a “rescue pathway”, allowing ions to be taken up if transport at the gill is impaired.

1.8 Synopsis

Recent declines in the whitebait fishery reveal the need for more research into how anthropogenic inputs threaten the stability of inanga populations. The aim of this research was to determine the accumulation and physiological impacts of trace elements on adult inanga. This study aimed to better understand ion transport in inanga, and to what extent metals interfere with this transport. By determining how the specific physiology of this fish impacts their sensitivity to trace elements, better environmental regulations can be developed.

The migratory behavior of inanga involves exercise stress through estuarine areas that may be sinks for contamination. Such stress has been shown to exacerbate Na^+ efflux in rainbow trout (Postlethwaite and McDonald, 1995). As such, the effect of handling stress on Na^+ transport in inanga was investigated in Chapter 3. Handling stress was used as a proxy

for any stressor that would increase gill perfusion (i.e. migration). The effect of stress on Cu accumulation was also examined, as Cu is a known competitive inhibitor for Na^+ uptake (Grosell and Wood, 2002), and thus factors which increase Na^+ influx may also alter Cu accumulation. Understanding how stress impacts metal accumulation is an important step in understanding how additional factors unaccounted for by the BLM, could alter the key relationships for which it is based.

The accumulation characteristics of trace elements in inanga were then examined in a range of contaminated streams through two *in situ* caging studies (Chapters 4 & 5). The initial study was a survey of metal contaminants across 34 streams on New Zealand's West Coast. This region has been affected by mining and has shown high levels of trace elements in previous studies (Greig et al., 2010; Harding, 2005). The first caging study specifically examined changes in inanga trace element body burdens in a small subset of streams. The second caging study was more in-depth, incorporating biomarkers (TBARS and NKA) of trace element exposure. Both of these caging studies revealed a relative resilience of inanga in terms of preventing trace element accumulation. These studies combined to provide an environmentally-realistic investigation of the relationship between trace element exposure and resulting body burdens in inanga.

Laboratory exposures of Fe and Zn were also performed on inanga (Chapter 6). Fe and Zn were used as they are both found in elevated levels in inanga habitats. These experiments allowed for the isolation of metal exposures from other unknown variables that may have interfered with accumulation and toxicity in the field. For comparison, one of the same biochemical biomarkers (TBARS) used in the caging study (Chapter 5) was also measured. Additional physiological endpoints of respiration rate, Ca^{2+} and Na^+ ion influx, and ammonia production were also measured after 96 h exposures. These supported *in situ* studies with no significant metal accumulation and little to no change in physiological and biochemical biomarker endpoints.

As trace element exposures often affect ion transport, the ability of inanga to transport ions across the skin was also a major aim of this thesis. Chapter 7 utilized multiple techniques in order to determine whether cutaneous ion transport occurred and its relative importance. Both Na^+ and Ca^{2+} transport rates were evaluated across the skin of inanga. Two methodologies were used, one *in vivo*, the other *in vitro*. *In vivo* partitioning experiments were used for both Na^+ and Ca^{2+} to determine the percent of transport

occurring across the body *versus* the head of the fish. Subsequent *in vitro* Ussing chamber techniques were then used to quantify the uptake of Ca^{2+} across inanga skin. The results of this chapter revealed a strong ability of inanga to transport ions across their skin, likely *via* transporters similar to those in the gill.

The implications of this research are discussed in Chapter 8. This includes the potential applications of this thesis to evaluate the applicability of the BLM in New Zealand and shape environmental policy. Understanding how inanga-specific physiology relates to trace element toxicology is a crucial step to protecting this important native fish.

Chapter 2: General Methods

2.1 Animal collection & husbandry

For all laboratory studies, adult inanga (*Galaxias maculatus*) were collected via seine netting in freshwater streams in the Canterbury region of New Zealand's South Island. Fish were transported under constant aeration to the University of Canterbury's aquarium facility, where they were transferred to 50-L plastic aquaria receiving flow-through freshwater (pH 6.8–7.2; [Na] 315–330 μM) at a fish density no greater than 2.5 kg m^{-3} . Water temperature was maintained at 11–15°C, with a photoperiod of 12L:12D. Fish were fed *ad libitum* with commercial flake food daily (NutrafinMax, USA), and allowed to acclimate to aquarium settings for at least 48 hours before any experimental manipulations.

2.2 Aquarium water

Aquarium water samples were sent to Hill Laboratories (Christchurch, New Zealand) for determination of water chemistry parameters (Table 2.1). Total and dissolved (filtered through a 0.45 μm syringe filter) trace element concentrations of aquarium water were determined via inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx; see Section 2.5) at the University of Canterbury (Table 2.2).

Table 2.1 Water chemistry results for aquarium water

Water parameter	Measurement
pH	6.7
Total Alkalinity (as CaCO ₃ equivalents)	52 mg L ⁻¹
Free CO ₂	21 mg L ⁻¹
Total Hardness (CaCO ₃)	70 mg L ⁻¹
Electrical Conductivity (EC)	18.8 mS m ⁻¹
Approximate Total Dissolved Salts	126 mg L ⁻¹
Total Boron	0.026 mg L ⁻¹
Total Calcium	23 mg L ⁻¹
Total Magnesium	2.9 mg L ⁻¹
Total Manganese	< 0.00053 mg L ⁻¹
Total Potassium	1.14 mg L ⁻¹
Total Sodium	8.6 mg L ⁻¹
Chloride	11 mg L ⁻¹
Nitrate	3.1 mg L ⁻¹
Sulphate	8.7 mg L ⁻¹

Table 2.2 Mean total and dissolved trace element concentrations (µg L⁻¹) ± standard error of mean for aquarium water as determined by ICP-MS. Cd and Co were below detection limits and were therefore excluded.

	Al	As	Cu	Cr	Fe	Ni	Pb	Zn
dissolved	0.63 ± 0.1	0.10 ± 0.01	1.0 ± 0.4	0.10 ± 0.01	1.12 ± 0.6	0.14 ± 0.01	0.06 ± 0.02	4.48 ± 0.2
total	4.16 ± 1.8	0.11 ± 0.01	2.1 ± 0.6	0.16 ± 0.04	12.27 ± 8.5	0.27 ± 0.07	0.29 ± 0.12	30.89 ± 23.9

2.3 Euthanasia and dissections

For the procedures detailed in Chapters 3 through 6, fish were euthanized via exposure to a lethal dose of 3-aminobenzoic acid ethylester (MS-222, 1 g L^{-1}) followed by spinal cord severance. Chapter 7 did not use anesthetics owing to the potential effect on these on membrane permeability. As such, these fish were caught, and euthanized *via* a blow to the head, with spinal cords subsequently severed to ensure euthanasia.

In Chapters 5 and 6, whole gill and liver samples were dissected for biomarker analyses following euthanasia. These samples were placed in 1.75 mL Eppendorf tubes and immediately flash frozen using liquid nitrogen. Samples were then stored in a -80°C freezer for biochemical assessments; see Section 2.8) for no more than 6 months. All animal manipulations were approved by the Animal Ethics Committee of the University of Canterbury (2012/36R).

2.4 Unidirectional ion flux

2.4.1 Ion influx

Ion influx rates were determined for inanga during handling stress experiments (Chapter 3) and after metal exposures (Chapter 6). Fish were placed in experimental containers, filled with water of varying chemistry depending on the experiment (see specific chapter methods), before isotope was added. Triplicate 1 mL initial water samples were taken for measurement of total water ion concentrations and radioactivity levels. After one hour, final triplicate 1 mL water samples were taken. Fish were then washed in a rinse containing high levels of the specific ion being measured and a lethal dose of 3-aminobenzoic acid ethylester (MS-222; 1 g L^{-1}). The high ion rinse was used to remove any isotope adsorbed to the body surface of the fish. This rinse was followed by a series of two distilled water rinses. Following spinal cord transection to ensure complete euthanasia, fish were blotted dry and weighed.

Water samples were then assessed for radioisotope concentrations using either a gamma counter for gamma-emitters ($^{22}\text{Na}^{+}$) or a liquid scintillation analyzer for beta-emitters ($^{45}\text{Ca}^{2+}$). Resulting cpm were then used to calculate specific activity ($\text{cpm } \mu\text{mole}^{-1}$):

Equation 2.1:

$$\text{Specific Activity} = \frac{\text{radiolabeled ion (cpm L}^{-1}\text{)}}{\text{ion concentration (}\mu\text{M)}}$$

where radiolabeled ion (cpm L⁻¹) is the counts per minute of the radiolabelled ion in the water and ion concentration (μM) is the total ion concentration in the water. This was then used to determine ion influx (J_{in} ; nmol g⁻¹ h⁻¹; Postlethwaite and McDonald, 1995):

Equation 2.2:

$$J_{in} = \frac{\text{fish radiolabeled ion (cpm)}}{SA * M * t}$$

where fish radiolabelled cpm is the counts per minute of the radiolabelled ion in exposed fish, SA is the specific activity of the water (cpm nmoles⁻¹), M is the mass of the fish in grams, and t is the duration in hours.

2.4.2 Na⁺ efflux

Na⁺ efflux rates were determined for handling stress experiments (Chapter 3). Fish were placed in experimental water (Na⁺-free, 1 mM Ca²⁺), which needed to be Na⁺ free in order to measure Na⁺ loss from fish. Triplicate 5-mL water samples were taken at time zero and every hour for the next three hours, for subsequent measurement of Na⁺ concentrations.

Concentrations of Na⁺ in water samples were determined *via* flame photometry. Na⁺ efflux rates (J_{out} ; μmol g⁻¹ h⁻¹) were then calculated using the following equation (Postlethwaite and McDonald, 1995):

Equation 2.3:

$$J_{out} = \frac{\Delta[Na]}{M * t}$$

Where Δ[Na] is the change in concentration of Na⁺ (μM), M is the mass of the fish in grams, and t is the duration in hours.

2.5 Caging exposures

Adult inanga were collected from a single source site, Orowaiti Lagoon (Oro), by setting gee-minnow traps overnight. Fish were then transported under constant aeration to the University of Canterbury's field station in Westport (5 min. drive) to recover for 24 hours in 50-L tanks filled with water from the lagoon. After recovery, sets of twenty fish were randomly assigned to individual 10-L buckets and driven, under constant aeration, to study sites.

Water chemistry parameters (pH, dissolved oxygen (DO), temperature, and salinity) were measured at each site. Salinity, pH, and temperature were measured *in situ* with a multimeter pH and conductivity meter (YSI Model 63). Additionally, water samples were taken for metal analysis and determination of NOM (mg C L^{-1} , see Section 2.6). Two water samples were collected in acid-washed containers at each site. One sample was filtered through 0.45 μm Millex mixed cellulose esters filters before both filtered (dissolved) and unfiltered (total) samples were acidified to $\text{pH} < 2$ with ultrapure nitric acid (approximately 50 μL per 10 mL water) within 24 hours. Samples were kept refrigerated at 5°C for a maximum of 2 weeks before analysis via ICP-MS.

Cages were constructed using 13 mm high-density polyethylene tubing and polyethylene shade cloth. The cages were cylindrical with approximate dimensions of 30 cm x 30 cm x 38 cm (Fig. 2.1). All materials were tested to ensure there was no leaching of metals prior to use by exposure to milli-Q, stream water, and 5% nitric acid for 10 days. The leachates were analyzed for Al, arsenic (As), Cd, Co, Cr, Cu, Fe, Ni, Pb, and Zn via ICP-MS, and no significant leaching of metals was determined. Twenty fish were placed in each cage (one cage per stream). Rocks from individual streams were placed at the bottom of each cage in order to anchor it in place, while polyethylene string was used to tie the cages to riparian vegetation with a small portion of the cage left exposed to air.

Up to ten fish, depending on survival, were collected at two time intervals at each stream. After collection, fish were euthanized (per Section 2.3) and subsampled. Fish were kept frozen at -20°C before digestion (Section 2.7.1) and determination of metal concentrations (Section 2.7.2).



Figure 2.1 Cylindrical cages constructed for *in situ* caging study (length = 38 cm, radius = 15 cm). Each cage contained 20 fish.

2.6 Natural organic matter (NOM)

NOM was measured using the methods of Glover *et al.* (2005). Solution absorbances at 300 nm (Unicam 8625 UV/Vis spectrometer) were converted to milligrams of C per liter using a previously established relationship between absorbance and NOM.

2.7 Trace element analyses

2.7.1 Sample digestions

Fish were weighed, freeze-dried in a FreeZone 2.5 L freeze-dryer (Labconco), broken apart by hand, and soaked in 1 mL of concentrated nitric acid and 4 mL of concentrated hydrochloric acid overnight before being digested in a heating block at approximately 80°C for 1 hour. Sediments were collected in acid-washed containers at each field site before

being dried in a 30°C oven for approximately one week. A subsample of 1 g for each stream was then digested with 4 mL 50% Analar grade nitric and 10 mL 20% Analar hydrochloric acid in a heating block at 85°C for 1 hour. All digests were brought to 20 mL volumes with ultrapure water. Blanks (ultrapure water) and certified reference materials (CRM) for both fish material (DORM-3, National Research Council Canada) and marine sediment (SRM 2702, National Institute of Standards & Technology, USA) were digested and run as quality controls.

2.7.2 ICP-MS

To prepare samples for ICP-MS, fish digests were diluted by adding 1 mL digest to 4 mL ultrapure water (for a 5x dilution). Gill and intestine digests were diluted by adding 0.5 mL of the digest to 4.5 mL of ultrapure (for a 10x dilution). Sediments were diluted by adding 0.5 mL digest to 10 mL 2% trace metal grade nitric acid (for a 21x dilution).

The ICP-MS was an Agilent 7500cx fitted with a collision cell. Samples were analyzed in collision mode with helium to remove polyatomic interferences and ^{103}Rh added on-line as the internal standard. Recoveries for Al, As, Cd, Co, Cr, Cu, Fe, and Ni in CRM averaged $75.9 \pm 2\%$; however, the recovery for Pb was below 50%. Calculating recoveries for CRM is an important quality control check, so to be sure the instrument was not at fault for low Pb values, an additional mussel CRM (SRM 2976, National Institute of Standards & Technology, USA) was also run, resulting in an 82.4% recovery. According to U.S. EPA standards, recoveries should fall between 80-120% of the true value for CRMs (US EPA, 1994). Detection limits for the instrument were $0.1 \mu\text{g L}^{-1}$ for most metals. Analytes with more than 50% of samples below calculated detection limits were not included in statistical analyses. When more than 50% of the samples had readings above detection limits, sample concentrations below detection limits were assigned a value of half the detection limit for that sample type.

2.8 Biochemical analyses

2.8.1 Lipid peroxidation

Measures of lipid peroxidation (LPO) were used as an indicator of oxidative stress in liver samples through the formation of TBARS. Malondialdehyde (MDA), a product of lipid peroxidation, was measured using its reaction with thiobarbituric acid (TBA) to form a colored product. The color resulting at 532 nm is proportional to the MDA present. Sigma-Aldrich lipid peroxidation kits (catalog number: MAK085) were used, and all samples and standards were run in duplicate.

Liver samples were weighed and homogenized on ice in 1.75 mL Eppendorf tubes using a pellet pestle in 800 μ L phosphate buffer (PBS) (1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 137 mM NaCl, pH 7.5). A standard curve for MDA was produced using the provided MDA stock from the kits. Standards were diluted from this stock to reach 0, 4, 8, 12, 16, and 20 nmoles MDA. A 200 μ L subsample of liver homogenate was added to 300 μ L of the provided lysis buffer and 3 μ L of butylated hydroxytoluene (BHT), mixed, and centrifuged at 13,000 \times g for 10 minutes. For both standard solutions and sample homogenates, 200 μ L was added to 600 μ L of TBA solution (reconstituted with 7.5 mL of glacial acetic acid and brought to 25 mL with distilled water). Homogenates with TBA were then incubated at 95°C for 60 minutes followed by 10 minutes in an ice bath to cool before 200 μ L aliquots were plated on 96 well microplates and read on a UV spectrophotometer (Molecular Devices, SpectraMax M5). The rate of MDA formation was calculated as nmol TBARS formed per milligram of protein (nmol TBARS mg protein⁻¹).

2.8.2 Na⁺/K⁺-ATPase (NKA)

NKA activity was measured in gill samples with a 96 well microplate assay using methods similar to McCormick (1993). Gill samples (processed 10 at a time) were weighed and homogenized using a plastic pellet pestle in 600 μ L of an ice-cold imidazole buffer (IB) (50 mM, pH 7.5). As the ouabain-sensitive hydrolysis of ATP is coupled to the reduction of nicotinamide adenine dinucleotide, oxidized (NAD^+) to nicotinamide adenine dinucleotide, reduced (NADH); the production of NADH measured at 340 nm directly relates to ATP

hydrolysis and therefore NKA activity. Ouabain acts to block ATP hydrolysis by NKA, so samples with ouabain are subtracted from those without to determine NKA activity. An ADP standard curve was run using adenosine 5' diphosphate disodium salt from a bacterial source. Two working solutions were made either with or without the addition of ouabain. Working solution A contained 14 mL stock A (2.8 mM phosphoenolpyruvate, 3.5 mM ATP, 0.22 mM NADH, 4 U mL⁻¹ lactate dehydrogenase, and 5 U mL⁻¹ pyruvate kinase), 5 mL salt solution (200 mM NaCl, 10.5 mM MgCl₂·6H₂O, and 50 mM KCl), and 1.42 mL of 50 mM IB. Working solution B contained the same amounts of stock A and salt solution, but included ouabain (3 mM). Four 10 µL subsamples of tissue homogenates were added to microplates followed by the addition of 200 µL of either the ouabain and ouabain-free solutions (two of each), and absorbance was read every 20 seconds for 5 minutes at 340 nm on the same UV spectrophotometer as used in Section 2.8.1. The resulting slope was used to calculate V_{\max} (maximum rate of the reaction) for the decay curve of NADH by subtracting the slope of inhibited samples (with ouabain) from those of uninhibited samples (without ouabain). Protein concentrations were determined via the Bradford (1976) assay (see Section 2.8.3), and enzyme activity was expressed as µmol ADP mg protein⁻¹ h⁻¹.

2.8.3 Bradford protein assay

Protein concentrations of both gill and liver samples were determined via the Bradford (1976) colorimetric assay in order to standardize LPO and NKA results (see Sections 2.8.1 and 2.8.2). The assay is based on the binding of a dye (Coomassie Brilliant blue G-250) to proteins, shifting the absorption of the dye from 465 to 595 nm where it is read. A standard curve was produced using 0, 5, 10, and 20 nmol bovine serum albumin (BSA). Liver and gill homogenates were centrifuged at 10,000 x g at 5°C for 5 min before analysis. Where necessary, samples were diluted in the same PBS buffer as Section 2.8.1 to bring them into range of the standard curve. Samples were run in triplicate, with 10 µL subsamples of homogenates and 200 µL Bradford reagent added to microplates, left in the dark for 5 minutes, and read at 595 nm on the same UV spectrophotometer as used in Section 2.8.1.

2.9 Exposures

2.9.1 Experimental setup

Laboratory Fe and Zn exposures were conducted in a 15°C temperature controlled room. Exposure containers (5 L plastic containers) were soaked in hot water for at least an hour, acid washed in 50% Analar nitric acid, rinsed with tap water, and dried before use. Containers were filled with 3 L of aquarium water, metals were added (see Section 6.2.2), and given 24 hours to equilibrate. Three fish were then added to each container of varying metal concentrations and covered with a plastic mesh. Exposures lasted for 96 hours and were under constant aeration. Approximately half of the water was changed after 48 hours, using similarly metal-spiked waters made the day before. Two 5 mL water samples were taken at the start of the experiment (time 0), before and after water changes, and at the termination of the exposures (96 h). One sample was acidified (50 µL trace grade nitric per 15 mL) for total metal concentrations, and the other was filtered through a 0.45 µm Millipore filter before acidification for dissolved metal concentrations. After 96 hours fish were removed for measurement of physiological and biochemical endpoints (see Sections 2.8.1, 2.8.2, 2.9.2, and 2.9.3).

2.9.2 Oxygen consumption

After 96 hours of metal exposure, fish were placed in closed-box respirometers (250 mL Schott glass bottles sealed with bungs). Oxygen uptake was measured using methods similar to Sloman et al. (2006). Respirometer temperatures were maintained at 15°C using a temperature controlled water bath. Water samples (approximately 1 mL) were drawn via syringe at the start and end of the procedure (time 0 and 1 hour). Partial pressure of oxygen (PO₂) was measured using a Strathkelvin oxygen electrode and meter (models 1302 and 781, respectively; Strathkelvin Instruments, Glasgow, Scotland). Oxygen consumption rates were calculated using the following equations (Cech, 1990):

Equation 2.4:

$$CO_{2(WS)} = \frac{PO_{2(WS)} * CO_{2(AS)}}{PO_{2(AS)}}$$

Where $CO_{2(WS)}$ = O_2 concentration ($mL O_2 L^{-1}$) in the water sample, $PO_{2(WS)}$ = O_2 tension (mmHg) in the water sample, $CO_{2(AS)}$ = O_2 concentration in water at air saturation (from nomogram or tables), and $PO_{2(AS)}$ = O_2 tension at air saturation (from Equation 2.5).

Equation 2.5:

$$PO_{2(AS)} = 0.2094(P_B - P_{WV})$$

Where 0.2094 = mole fraction or volumetric fraction of O_2 in the atmosphere, P_B = total barometric pressure (mmHg), and P_{WV} = water vapor pressure at the experimental temperature (mmHg). Oxygen was converted from $mL O_2 L^{-1}$ to $mg O_2 L^{-1}$ ($1 mg O_2 = 1.428 mL O_2$), and oxygen consumption (MO_2 , $\mu mol g^{-1} h^{-1}$) was calculated using the following equation (Cech, 1990):

Equation 2.6:

$$MO_2 = \frac{(CO_{2(A)} - CO_{2(B)})V}{T * M}$$

Where MO_2 = O_2 consumption rate ($\mu mol O_2 g^{-1} h^{-1}$), $CO_{2(A)}$ = O_2 concentration in water ($mg O_2 L^{-1}$) at the start of the measurement period, $CO_{2(B)}$ = O_2 concentration in water ($mg O_2 L^{-1}$) at the end of the measurement period, V = volume of respirometer (L), M = mass in g, and T = time elapsed during measurement period (h).

Before each use, electrodes were calibrated using a sodium tetraborate solution as a zero and oxygen saturated water as the maximum. Blank respirometers were run as a control with each trial in order to account for any microbial respiration in the water.

2.9.3 Ammonia

Water samples (1 mL) were taken from respiration chambers (Section 2.9.2) before and after initial and final respiration samples, respectively. Samples were used to measure ammonia production via the salicylate method (API Ammonia Test Kit). In this method, ammonia reacts with salicylate and hypochlorite ions in the presence of ferricyanide ions to form the salicylic acid analog of indophenol blue. Absorbance at 650 nm is then directly proportional to the concentration of ammonia present (Solorzano, 1969). Initially, approximately 100 μL of salicylate was added to the 1.25 mL water sample and mixed before approximately 100 μL of the hypochlorite/ferricyanide solution was added, mixed, and left for at least 5 minutes to develop. Samples were then read at 650 nm on a visible spectrophotometer (Novaspec III, Amersham Biosciences).

2.10 Ussing chamber

Fish were euthanized via spinal cord severance before skin was dissected and mounted on chamber inserts for a dual channel Ussing chamber (Warner Instruments model U-2500). Skin mounted on chamber inserts was placed into the Ussing device and bathing solutions placed on either side, the composition of which varied depending on the procedure (see Chapter 7). These solutions were bubbled with 0.3% CO_2 in oxygen for the duration of the experiments. One side was spiked with 10-20 μL $^{45}\text{Ca}^{2+}$ stock (1-2 μCi) and 0.5 mL samples were taken after 5 min (time 0) and each hour after for 2 hours. Samples were replaced with 0.5 mL of corresponding solutions and corrected for in flux calculations. Various metals and pharmacological agents (Fe, Zn, La, and verapamil) were also added to determine effects on Ca uptake rates. Serosal and mucosal water samples were then run for ^{45}Ca on the liquid scintillation analyzer (Perkin Elmer Tricarb 2910 TR), following addition of scintillation fluor. Resulting cpm were used to calculate specific activity *via* Equation 2.1, and Ca uptake ($\text{nmol cm}^{-2} \text{ h}^{-1}$) *via* Equation 2.2 (where the mass of the fish was replaced with the surface area of skin exposed).

2.11 Statistical analyses

All data in the thesis was subjected to tests for the assumptions of parametric statistical analyses. Normality was tested using the Shapiro-Wilk test and homoscedasticity was tested using Bartlett's Test. When necessary, data were log-transformed to ensure assumptions of normality were met. If data were normal they were analyzed using parametric statistical tests (usually a Student's t-test or ANOVA). Tukey's Honest Significance Difference (HSD) post-hoc tests were used to determine significance between specific groups. If parametric assumptions could not be met through transformations, a non-parametric Kruskal-Wallis test was used. The false discovery rate (FDR) method was used when p-value adjustments were necessary (after multiple comparisons), as it has been acknowledged as the best compromise for minimizing both Type I and Type II errors (Nakagawa, 2004; Verhoeven et al., 2005). For all tests, a $p < 0.05$ was considered significant.

Chapter 3: The impacts of stress on sodium metabolism and copper accumulation in a freshwater fish

This chapter has previously appeared in a modified form:

Harley, Rachel A., Glover, Chris N., 2014. The impacts of stress on sodium metabolism and copper accumulation in a freshwater fish. *Aquatic Toxicology* 147, 41-47.

3.1 Introduction

Stress has been shown to result in the inhibition of reproduction, growth, and the immune system in multiple fish species (McDonald and Milligan, 1997; Postlethwaite and McDonald, 1995). The hormones cortisol and adrenaline are released in response to a stressful event, leading to a host of physiological effects; including increases in heart rate and the permeability of the gills (Eddy and Handy, 2012; Wendelaar Bonga, 1997). Specific responses, however, will vary according to species and the magnitude/duration of the applied stress (Barton, 2002).

In terms of ion homeostasis, stress is thought to increase the functional surface area of the gills, and subsequently increase the rates of diffusional ion loss (Gonzalez and McDonald, 1992; Randall et al., 1972). Specifically, handling stress has been shown to result in large Na^+ losses, leading to a decrease in whole body Na^+ (Postlethwaite and McDonald, 1995). This diffusive loss of Na^+ creates an internal ion deficiency that is energetically costly to restore (McDonald and Milligan, 1997). Ventilation frequency has also been shown to increase in response to stress. For example, fish from low-predation areas have a lower ventilatory frequency under normal conditions than those from high-predation areas (Brown et al., 2005). This response, combined with elevated gill permeability, exacerbates exchange between the external environment and the blood, resulting in elevated ion transport rates (Cairns et al., 1982; Hughes and Roberts, 1970). Na^+ transport rates are also thought to scale with size, with smaller fish showing higher influx rates owing to the larger surface area to body mass ratio (Grosell et al., 2002). Because of this, small fish may exhibit a more exaggerated Na^+ ion response to stress (McDonald and Milligan, 1997).

The migratory behavior of inanga means they must swim through potentially contaminated estuaries in order to reach adult freshwater habitats. Elevated levels of metal contaminants have been found in many of these migratory streams along New Zealand's West Coast (Greig et al., 2010). As a consequence, inanga will be exposed to toxicants under conditions where gill perfusion and ventilation is increased. As such, ion loss would be expected to increase, leading to an enhanced compensatory ion uptake. This could result in the possible increase in uptake of ion-mimicking toxicants such as Cu.

Na^+ absorption is essential in balancing diffusive Na^+ loss in freshwater fish. Cu is thought to compete with Na^+ for absorption at the apical surface of gill epithelium (Grosell

and Wood, 2002), although the exact nature of the transporter (i.e. ENaC, NHE, and/or Rh protein metabolon) remains controversial. At high concentrations, Cu is thought to displace Ca^{2+} in tight junctions leading to an increase in gill permeability and Na^+ efflux (Grosell and Wood, 2002). In addition, Cu is also thought to impair NKA activity on the basolateral membrane of the gills, resulting in an impaired ability to absorb Na^+ ; owing to the proposed role of this enzyme in generating electrochemical gradients that drive Na^+ absorption (Grosell et al., 2007). Hence, Na^+ balance may be more difficult to restore. Understanding how stress can influence ion transport and resulting contaminant uptake could provide important information regarding the environmental susceptibility of inanga and fish with similar physiology and behavior.

This study aimed to evaluate the effects of handling stress on ventilation and Na^+ metabolism in inanga. The working hypothesis examined was that handling stress would lead to higher Na^+ influx and therefore higher Cu accumulation. Specifically, Na^+ influx and efflux were evaluated directly after handling and subsequently following a 24-hour recovery period. The effect of handling stress on Cu absorption was also evaluated by measuring total body Cu levels following similar handling treatments. Quantifying the effect of stress is important in establishing acclimation periods necessary for laboratory studies examining branchial physiology; given that processes such as ion transport, toxicant uptake, waste excretion, and oxygen consumption are all located at the gills, and are all likely to be modified by ventilation rates. Furthermore, the impact of stress on gill ventilation and perfusion may alter metal handling, possibly leading to increased susceptibility to waterborne contaminants taken up by the branchial epithelium. This has been previously observed for organic toxicants such as ethinylestradiol in swimming killifish (Blewett et al., 2013). Consequently, an understanding of how stress impacts physiology and toxicant accumulation is important in terms of risk assessment, conservation, and maintenance of a sustainable inanga fishery.

3.2 Methods

Fish collection and animal husbandry followed methods described in Section 2.1. Inanga were exposed to handling stress at the start of each experiment through capture by

mesh netting, transport in a 5-L bucket and re-handling during setup. All procedures were performed in a temperature controlled room at 15°C.

3.2.1 Na⁺ influx

Na⁺ influx rates were assessed using methods similar to Glover et al. (2012). Individual inanga (n = 6; mass range 0.4-1.2 g) were exposed to handling stress before being placed in separate 250 mL containers of Na⁺-free experimental water consisting of 1 mM Ca²⁺ (added as calcium acetate) and pH adjusted to 7 by adding either KOH or HCl (Glover et al., 2012). This water chemistry was chosen to match that used in efflux experiments (Section 3.2.2). In order to determine the impact of water chemistry on influx, an identical trial was also conducted using aquarium water (pH 7; [Na] approx. 430 nM). Water was then spiked with 10 µl of ²²Na⁺ isotope (~5 µCi L⁻¹; Perkin Elmer) before samples were collected as described in Section 2.4.1. The high Na⁺ rinse contained 1 M NaCl. Whole fish and water samples were measured for radiolabelled Na⁺ incorporation via gamma counting (Wallac Wizard 1470; Perkin Elmer). Water samples were analyzed for Na⁺ via flame photometry (Sherwood Instruments). Na⁺ influx was then calculated using Equations 2.1 & 2.2 (Section 2.4.1).

A 'no handling' group (n = 6 individual fish) was treated under identical protocols to those described above with the exception that these fish were covered with netting, submerged in their exposure containers in aquarium water, and left overnight (24 hours). To minimize disturbance to the fish, water was carefully drained from each individual container using a needle point siphon until an approximate volume of 250 mL remained in each experimental container. Containers were then spiked with isotope and experiments proceeded as above.

3.2.2 Na⁺ efflux

Na⁺ efflux rates were also assessed in response to handling stress using methods similar to Glover et al. (2012). Fish were exposed to handling stress in a manner identical to that of influx trials, although rates were measured both as individuals and in groups. This

was to determine any effect of grouping on the stress response, as inanga are schooling fish (Mitchell, 1989) and therefore may exhibit less stress in groups.

For grouped efflux experiments, about 18 fish (total biomass ranging from 1.7 g L⁻¹ to 3.1 g L⁻¹) were placed in a 10 L tank (n = 3) with approximately 5.5-7 L of experimental water, with a composition identical to influx trials (Na⁺-free, 1 mM Ca²⁺). Fish were initially left overnight in aquarium water to acclimate. After 24 hours, fresh aquarium water was added to gently flush out experimental tanks while minimizing stress to the fish. Once the overnight water had been adequately flushed, a new volume of experimental water was added to the exposure tanks and sampling was undertaken. Water samples were taken at time zero and every hour for the next three hours. Water samples were then analyzed and efflux calculated as described in Section 2.4.2 (Equations 2.1 & 2.3).

For individual efflux experiments, seven individual inanga (mass ranging from 0.07-1.40 g) were placed in separate containers filled with 250 mL of experimental water (composition as above) after handling stress. Water sample collection and handling were similar to those described in influx trials (Section 3.2.1), and efflux was calculated as described in Section 2.4.2 (Equations 2.1 & 2.3).

During individual fish efflux trials, opercular frequency was also determined for each of the seven fish for 60 seconds at the start of each hour, for a total of four counts per fish. This was done immediately after handling and 24 hours later.

3.2.3 Cu exposure

To determine the effect of handling stress on Cu influx, an identical experimental design to that described for Na⁺ influx was used; with the exception that *in lieu* of Na⁺ isotope, Cu was added to a final concentration of 500 µg L⁻¹ from a freshly-prepared stock of CuSO₄. Six individual fish were placed into separate containers filled with 250 mL of Cu-contaminated experimental water immediately after handling, while the 'no handling' group were left for 24 hours before the water was spiked with Cu. Following euthanasia, fish were acid digested with 6 mL concentrated trace grade nitric acid (70% HNO₃) on a hot plate for approximately 30 minutes. Digests were then brought to approximately 20 mL with ultrapure (>18 MΩ) water and analyzed for total Cu concentrations using graphite furnace atomic absorption spectrophotometry (GBC Avanta Sigma Series).

3.2.4 Statistical analyses

Differences in Na^+ influx rates (J_{in}) and Cu accumulation between "stressed" and "unstressed" fish were evaluated using a Student's t-test, while individual and group efflux rates (J_{out}) were evaluated using a two-way analysis of variance (ANOVA) using stress status and time as the two factors. Opercular frequency was evaluated with two-way repeated measures ANOVA, with stress status and time as the two factors. Holm-Sidak post-hoc pairwise multiple comparisons were then used to assess differences across time points. Mean efflux rates were related to opercular frequency using simple linear regression. Cu absorption values were log transformed and efflux rates for individuals were square root transformed in order to achieve equal variances. All statistical analyses were conducted using SigmaStat, with a significance level of $p < 0.05$.

3.3 Results

3.3.1 Na^+ influx

Na^+ influx rates ranged from 774 to 1840 $\text{nmol g}^{-1} \text{h}^{-1}$ on the same day of handling (in both water types), and from 355 to 907 $\text{nmol g}^{-1} \text{h}^{-1}$ after the 24 hour acclimation period. Mean Na^+ influx rates were significantly higher on the same day of handling (Day 1, aquarium water) than 24 hours after handling (Day 2, aquarium water) (t-test, $p = 0.007$, Fig. 3.1). Water type (experimental *versus* aquarium) did not have a significant impact on influx rates (t-test, $p = 0.073$, Fig. 3.1). Mean influx rates on Day 1 were more than twice those of Day 2 (Fig. 3.1), revealing an increase in Na^+ influx rates in response to handling stress.

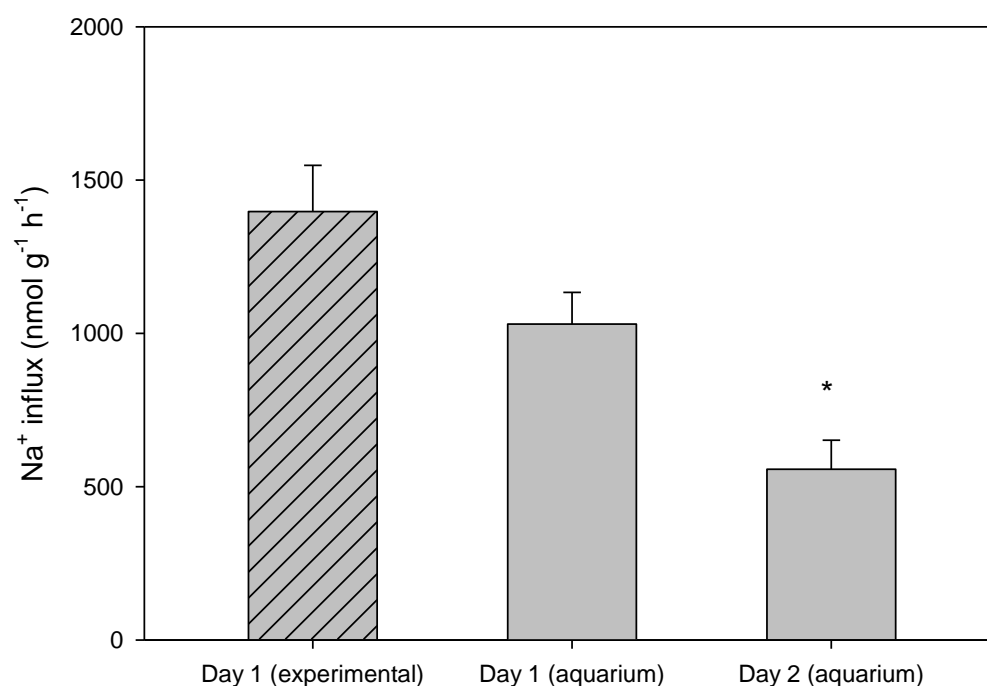


Figure 3.1 Mean Na⁺ influx rates (J_{in} ; nmol g⁻¹ h⁻¹) for individual (n=6) inanga (*Galaxias maculatus*) after handling in experimental water (Day 1), aquarium water (Day 1), and after a 24 hour acclimation period in aquarium water (Day 2). Error bars represent one standard error from the mean. Asterisk denotes a significant difference between treatments (Day 1 and Day 2), as determined by Student's t-test ($\alpha = 0.05$).

3.3.2 Na⁺ efflux

Mean Na⁺ efflux rates for individual inanga were highest directly after handling stress, with a significant decrease after the first hour (Holm-Sidak pairwise comparison, $p < 0.001$), followed by a further decrease in the next hour (Fig. 3.2). Efflux rates were significantly higher on Day 1 than those following the 24 hour acclimation period (two-way ANOVA, $p < 0.001$), with rates more than 5 times higher directly after handling (Fig. 3.2).

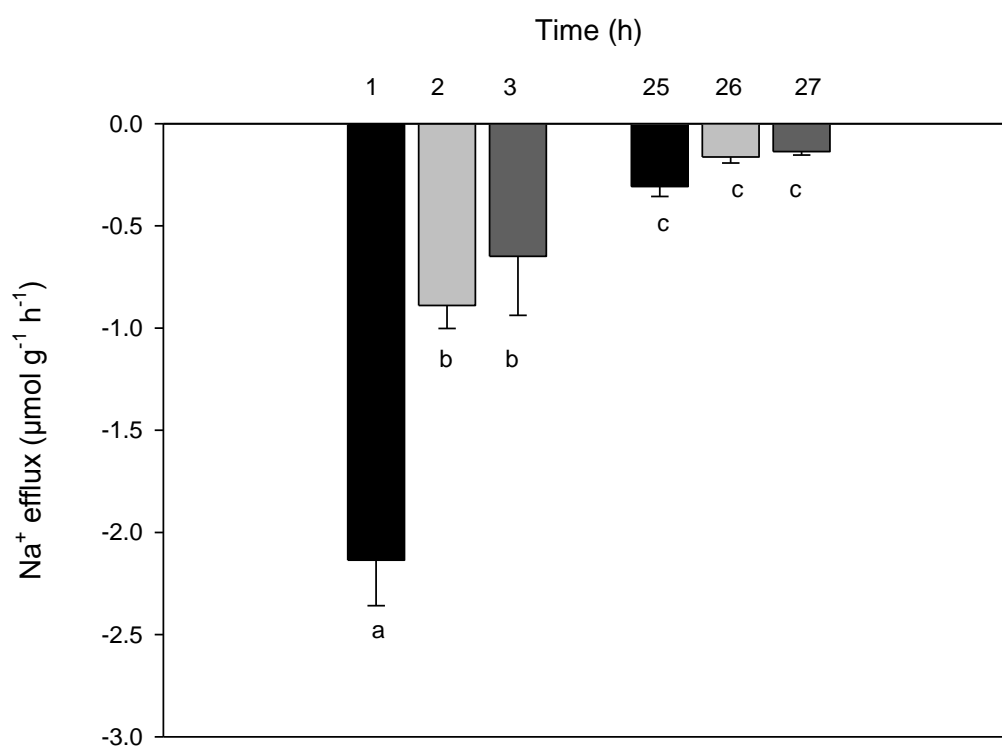


Figure 3.2 Mean Na⁺ efflux rates (J_{out} ; $\mu\text{mol g}^{-1} \text{h}^{-1}$) for individual ($n=7$) inanga (*Galaxias maculatus*) on the same day of handling (Day 1) and 24 hours later (Day 2). Error bars represent one standard error from the mean. Letters indicate significant differences between treatment means as determined by a two-way ANOVA followed by Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

Similar to individual fish, grouped inanga exhibited an elevated Na^+ efflux rate directly after handling, with rates in the first hour more than twice that of the following hour (Fig. 3.3). Efflux rates on Day 1 were significantly higher than those following the 24 hour acclimation period (two-way ANOVA, $p = 0.050$). However, groups showed higher variation relative to individuals in terms of recovery, with efflux rates on hour 3 similar to initial rates (Fig. 3.3).

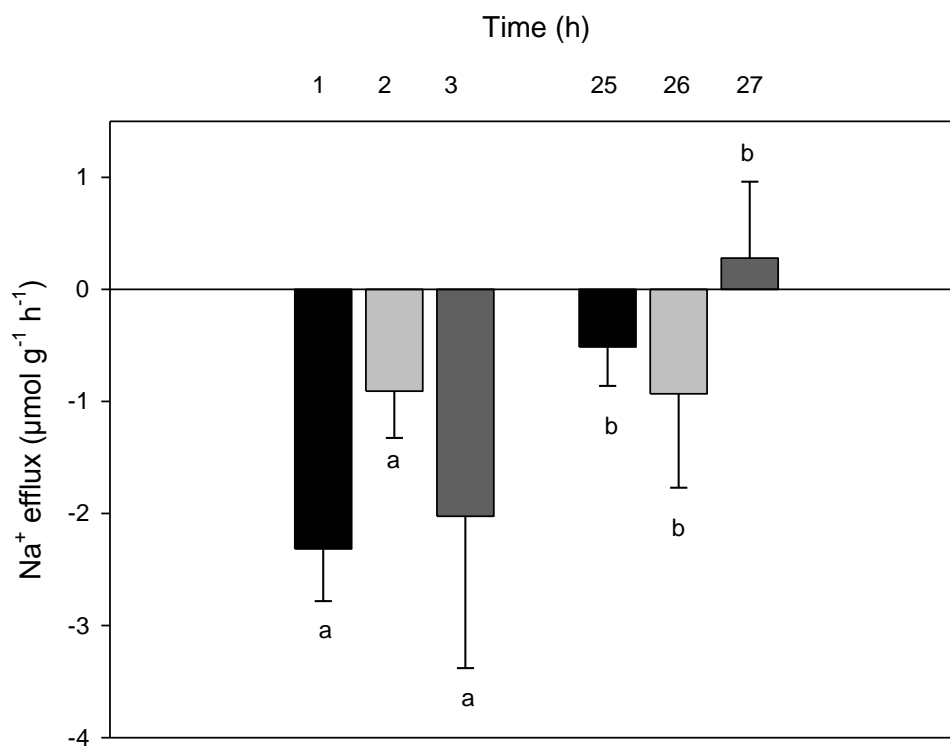


Figure 3.3 Mean Na^+ efflux rates (J_{out} ; $\mu\text{mol g}^{-1} \text{h}^{-1}$) for grouped ($n=3$) inanga (*Galaxias maculatus*) on the same day of handling (Day 1) and 24 hours later (Day 2). Error bars represent one standard error from the mean. Different letters indicate significant differences between treatment means as determined by a two-way ANOVA followed by Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

Overall, Na^+ efflux was statistically increased in response to handling stress in both individual and grouped fish. In both groups, efflux rates were decreased by more than half after the third hour (Fig 3.2 & 3.3). Overall, the efflux response appeared to be largely alleviated within 24 hours.

3.3.3 Opercular frequency

Mean opercular frequencies during individual efflux trials are presented in Fig. 3.4. There was a clear decrease in opercular frequencies over the three hour period on both days. Day 2 appeared to show a slightly steeper decrease than Day 1, but differences between days just eluded significance (two-way RM ANOVA, $p = 0.056$). Initial rates were similar between Days 1 and 2 (approximately $75 \text{ beats min}^{-1}$, Fig. 3.4), likely due to initial disturbances related to the observer. After the fish grew accustomed to the proximity of the observer, opercular frequencies decreased (hours 1-3). Holm-Sidak pairwise multiple comparisons revealed significant differences between initial frequencies (hour 0) and subsequent hours (hours 1, 2, and 3) (each $p < 0.01$) for both treatments.

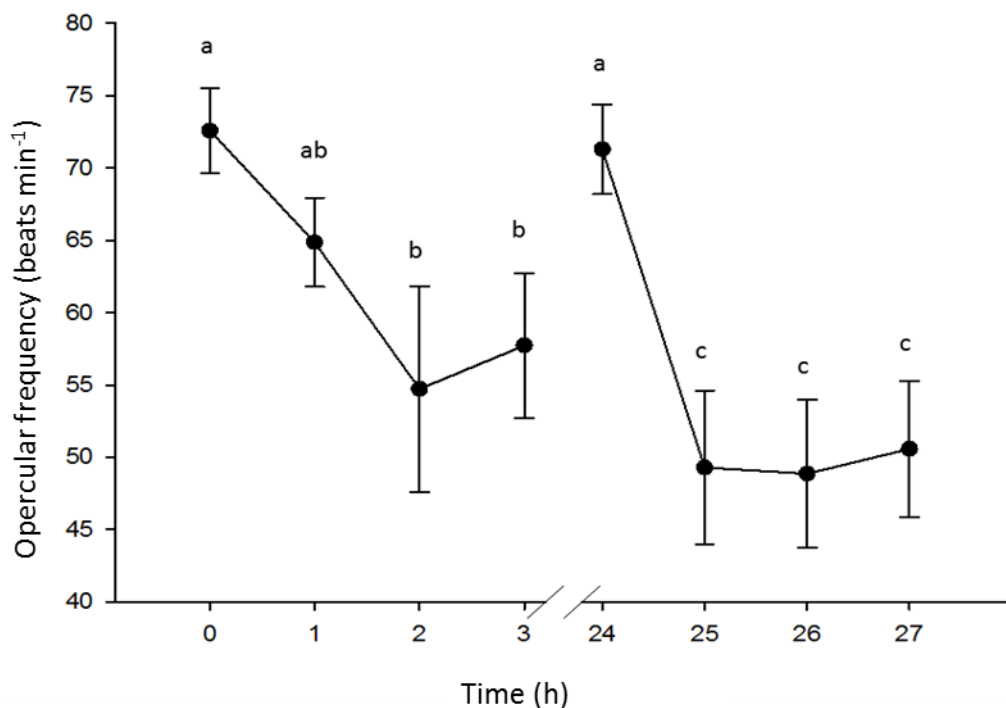


Figure 3.4 Mean opercular frequency (beats min⁻¹), $n=7$, directly after handling (Day 1 – Time 0-3) and 24 hours later (Day 2 – Time 24-27). Error bars represent one standard error from the mean. Different letters indicate significant differences between treatment means as determined by a two-way RM ANOVA and Holm-Sidak pairwise comparisons ($\alpha = 0.05$).

There was a positive significant relationship between efflux rates and opercular frequency (Fig. 3.5). As opercular frequency increased, efflux rates increased linearly ($r^2 = 0.8706$). As such, a large amount of the variance in efflux rates could be predicted by the variance in opercular frequency.

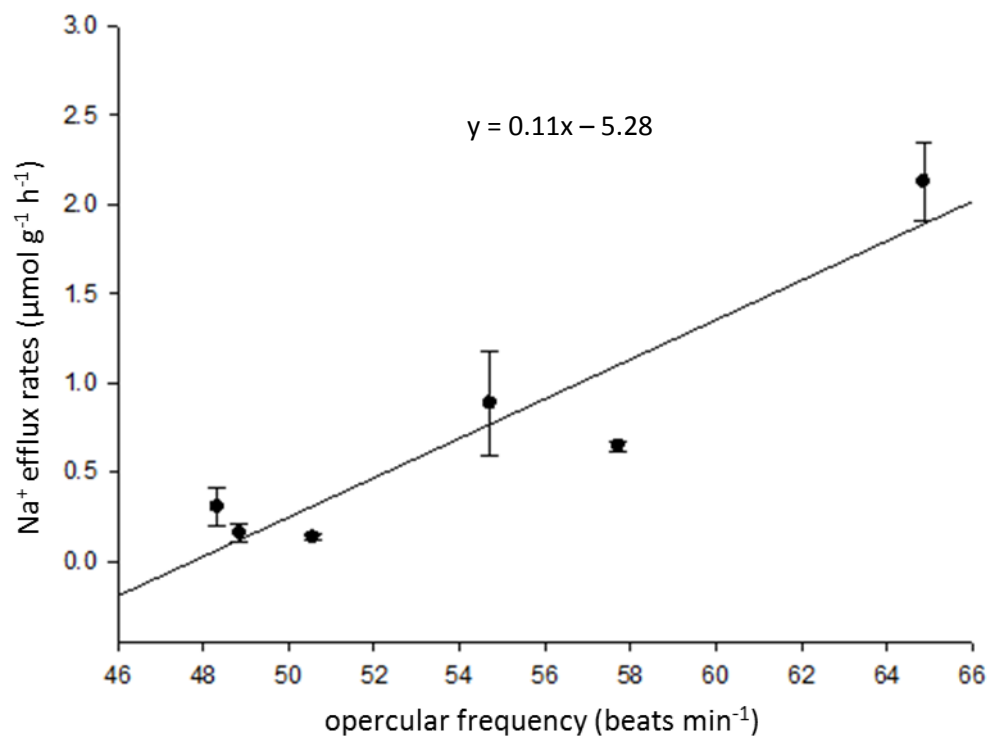


Figure 3.5 Linear regression for mean opercular frequency (beats min⁻¹) and mean individual Na⁺ efflux rates (μmol g⁻¹ h⁻¹) ($r^2 = 0.8706$).

3.3.4 Cu exposure

Total body Cu accumulated by inanga over a one hour exposure, following similar handling treatments, is exhibited in Figure 3.5. These data show that Cu accumulation was significantly higher in fish on the same day of handling compared to fish after the 24 hour acclimation period (Fig. 3.6, t-test, $p < 0.05$).

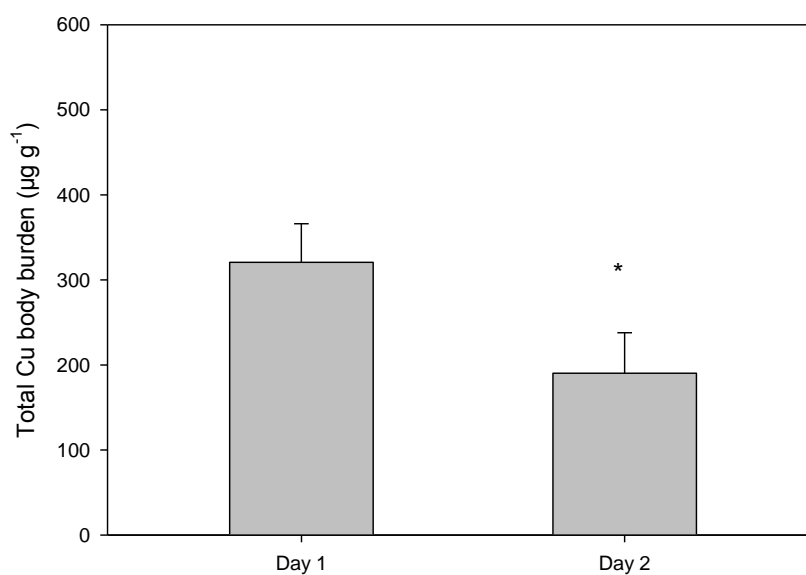


Figure 3.6 Total Cu body burden in individual fish exposed to $500 \mu\text{g L}^{-1}$ CuSO_4 for one hour on the same day of handling stress (Day 1, $n = 6$) and 24 hours later (Day 2, $n = 6$). Error bars represent one standard error from the mean. Significant difference in the mean denoted with asterisks as determined by a Student's t-test ($\alpha = 0.05$).

3.4 Discussion

3.4.1 Na⁺ metabolism

Stress in freshwater fish has been shown to increase ventilatory frequency and gill membrane permeability (Barreto and Volpato, 2006; Wendelaar Bonga, 1997). These changes lead to more lamellae being perfused, an increase in water influx across the gills, and an exacerbated Na⁺ efflux (McDonald and Milligan, 1997; Postlethwaite and McDonald, 1995; Wood and Randall, 1973). Consequently, Na⁺ ion absorption is enhanced in order to compensate for this loss. The current study supports these mechanisms, with handling stress leading to increased ventilation, exacerbated Na⁺ loss, and compensatory Na⁺ influx.

An increase in Na⁺ influx in response to enhanced Na⁺ efflux would seem necessary to balance Na⁺ homeostasis, however not all fish appear to exhibit such a response. Swimming rainbow trout, for example, showed a 70% elevation in Na⁺ efflux but did not exhibit any changes in Na⁺ influx (Wood and Randall, 1973). A rapid compensatory elevation in Na⁺ influx in inanga suggests plasticity of specific membrane transport pathways. The exact mechanisms of Na⁺ uptake across the apical membrane of the fish gill have yet to be fully elucidated. Multiple mechanisms have been proposed to explain this transport (see Section 1.3), and thus the specific mechanism by which Na⁺ influx is stimulated in inanga requires further examination. However, it is possible that their ability to rapidly compensate relates to their amphidromous lifestyle. The capability of inanga to move freely between waters of different salinities suggests a highly plastic cellular Na⁺ transport pathway. Indeed, a previous study has shown that these fish switch NKA subunit isoforms in response to rapid salinity change, a mechanism that conferred distinct transport characteristics (Urbina et al., 2013).

In contrast to variable influx responses to enhanced gill ventilation in fish, an enhanced Na⁺ efflux appears more conserved; however, the magnitude appears to differ. For example, Postlethwaite and McDonald (1995) found a 1.5-fold increase in Na⁺ efflux after exercise stress in rainbow trout; a rate that returned to control levels after 30 minutes. In the current study, inanga displayed stressed efflux rates in the range of 5-6 times 'recovered' efflux rates. This suggests that both species and the type/duration of the stressor will determine the magnitude of this response. Fish used in the Postlethwaite and

McDonald (1995) study were also significantly larger than the fish used in the current study (6-25 g versus 0.4-1.4 g, respectively), supporting the concept that Na^+ metabolism scales with size (Grosell et al., 2002). Despite the difference in magnitude of the effect, it is likely that the mechanism of enhanced efflux is similar. It is proposed that ion efflux increases are a result of increased Na^+ permeability due to transcellular and/or paracellular leakage (Grosell et al., 2002).

In their natural habitats, inanga are schooling fish (Mitchell, 1989). Consequently, it was anticipated that the stress response in groups would be different from that displayed in individuals. For example, recent evidence has shown that zebrafish, another naturally schooling species, exhibit lower levels of the stress hormone cortisol when housed individually than in groups (Parker et al., 2012). However, there was no difference in the response observed between grouped and individual inanga, in that they both had lower efflux rates 24 h after handling (Fig. 3.2 & 3.3). This suggests that schooling may not be an important factor in the Na^+ efflux response to stress. This also has implications from an ethics perspective, as the findings indicate that inanga are not unduly stressed when held as individuals; and also suggests that individuals, rather than schools (see Urbina et al., 2011), are suitable experimental units, permitting reduced animal use.

Opercular frequency is a sensitive indicator that has been shown to increase in response to stress in a number of studies (Brown et al., 2005; Cairns et al., 1982; Hughes and Roberts, 1970). In the current study it was significantly correlated with increased Na^+ efflux (Fig. 3.4b). As opercular frequency increases, fresh water is swept across gill epithelia. This refreshes the diffusion gradient for Na^+ , leading to an increase in efflux as Na^+ moves down its concentration gradient (McDonald and Milligan, 1997). Opercular frequency is thought to increase as a result of stress due to enhanced metabolic demands allowing increased oxygen uptake to fuel a "fight or flight" response (Dalla Valle et al., 2003). The increase in opercular frequency is usually a transient phenomenon, and for most fish species, rates return to resting values within a few hours (Artigas et al., 2005; Olla et al., 1992; Papoutsoglou et al., 1999). This pattern was also observed in the present study regarding inanga. However, although the opercular frequency response of inanga was transient, there was also clear evidence of an observer effect. Opercular frequency was measured every hour for three hours in both "stressed" and "unstressed" fish. Results in Figure 3.4b clearly show an increase at the start of each measurement period. This suggests

that the mere presence of the observer was enough to stress the fish. However, the overall trend was a decrease in stress with time following handling (Fig. 3.4a).

Water chemistry appeared to have little effect on Na^+ metabolism as the composition of the water did not significantly affect ion flux. The nature of the efflux experiments meant that a Na^+ -free water needed to be used. Thus, for these trials experimental water was made with purified laboratory water, and the selective addition of Ca^{2+} ions. This water was identical in composition to that used for previous investigation of inanga Na^+ metabolism (Glover et al., 2012). For consistency, this efflux water chemistry was also used for influx trials. To ensure that water chemistry was not impacting results, Na^+ influx was also tested in natural water. The lack of any significant difference between the two water types, suggested water chemistry was not an important factor, and that the predominant effect observed was due to handling stress.

3.4.2 Cu accumulation

In the current study, stressed inanga exhibited a 59% increase in whole body Cu relative to unstressed fish (Fig. 3.5). The exact mechanism by which Cu accumulation is elevated in stressed inanga is not known. Presumably, however, there are two factors involved. The increased water flow over the gills would expose the epithelial surface to more Cu than the gills of unstressed fish, thus effectively increasing Cu bioavailability. This mechanism of enhanced toxicant uptake has been previously observed for organic toxicants that passively diffuse across gill epithelia (e.g. Blewett et al., 2013).

A second possible mechanism of enhanced Cu accumulation relates specifically to the branchial Cu absorption pathway. Cu is a well described Na^+ absorption antagonist in other fish species, and competes with Na^+ for apical entry into gill epithelial cells via Na^+ transporters (Grosell and Wood, 2002). In the present study, stress was shown to increase Na^+ influx, likely in order to maintain Na^+ homeostasis in response to exacerbated Na^+ efflux. As described above, this effect was likely mediated by specific changes to the Na^+ uptake pathway (be it either an increase in uptake capacity (i.e. more Na^+ transporters) and/or an increase in uptake affinity). This study supports the possibility of a shared pathway between Cu and Na^+ in inanga, although further studies would be required to confirm this.

Increased Cu accumulation in fish as a result of stress is an important finding. Short-term Cu accumulation has been previously shown to be a strong predictor of acute Cu toxicity (MacRae et al., 1999). In fact, this relationship is the basis of the BLM (see Section 1.4); a modelling tool that predicts toxicity based on prevailing water chemistry, which in turn largely determines Cu accumulation (Di Toro et al., 2001). As such, the results of the current study indicate that more Cu will be taken up when a fish is stressed, making it more susceptible to the toxic effects of Cu. This is particularly problematic given that the mode of Cu toxicity is via impairment of Na^+ homeostasis (Grosell and Wood, 2002). Specifically, Cu is thought to exert its toxicity by interfering with Na^+ absorption at the gills. This occurs via competitive inhibition at the apical surface and/or via inhibition of key enzymes involved in branchial ion homeostasis, such as NKA (Grosell and Wood, 2002). This causes hyponatremia, a reduced plasma volume, increased hematocrit, and increased blood viscosity (Wilson and Taylor, 1993). If severe enough, these changes can lead to cardiovascular collapse and death. Given that both stress and Cu toxicity impact Na^+ homeostasis, it suggests the potential for synergistic effects beyond those of the individual factors.

3.4.3 Environmental implications

Juvenile inanga migrate from the open ocean to freshwater streams, primarily occupying waters near the saltwater wedge (McDowall, 1989). These streams are often contaminated with metals such as Cu. For example, acid mine drainage along New Zealand's West Coast can result in Cu levels as high as $15 \mu\text{g L}^{-1}$ (Chapters 4 & 5). Additionally, urban streams subjected to storm flow in Canterbury, New Zealand exhibit mean total Cu levels of $16 \mu\text{g L}^{-1}$ (O'Sullivan et al., 2012). The concentrations of Cu known to cause toxicity to inanga and other galaxiid fish are unknown, however, these exposure concentrations encompass some reported 96 h LC_{50} values for salmonids (e.g. $2.8 \mu\text{g L}^{-1}$ for 2.7 g rainbow trout in low hardness water; Cusimano et al., 1986), a closely related group (McDowall, 2002). The current Australian/New Zealand water quality guidelines provide a trigger value of $1.4 \mu\text{g L}^{-1}$ for protection of 90% of freshwater species (ANZECC, 2000).

This study supports the conclusion that stress will lead to an increased Na^+ efflux, and subsequent Na^+ influx, in freshwater fish. Handling stress, although very short term, was

shown to cause a significant increase in both Na^+ influx and efflux; with a relatively long recovery period when compared to the duration of the stressor. Additionally, the increased Cu uptake during periods of stress has important conservation implications. However, there are likely species differences in terms of the magnitude and duration of this response and subsequent recovery. For this reason, these results may not be applicable to all fish species in contaminated waters.

Chapter 4: Assessment of trace element accumulation in inanga using an *in situ* caging study

This chapter has been submitted (27 Feb 2015) for publication in a modified form to Marine and Freshwater Research

4.1 Introduction

The West Coast of New Zealand's South Island is a particularly important region for the whitebait fishery. However, this region has been shown to have increased metal loads in downstream catchments as a result of runoff from both mining and agriculture (Greig et al., 2010; Harding, 2005; Wang et al., 2004; Winterbourn, 1998). Mine drainage can also lead to the acidification of nearby streams, thereby increasing the bioavailability of certain metals to aquatic biota (Greig et al., 2010). Increased bioavailability can enhance metal accumulation in resident fish, which can result in a wide range of effects including reduced growth rates, impaired immune function, and even mortality (Dalzell and MacFarlane, 1999; Kraemer et al., 2005a). Understanding the relationship between stream chemistry, environmental metal concentrations and trace metal accumulation in inanga is an important step in establishing the sensitivity of this fish to trace elements, and in turn ensuring adequate protection for this important species.

Water chemistry is a significant parameter influencing metal speciation, and therefore bioavailability of metals to freshwater organisms (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2002). The effects of these factors on trace element toxicity are described in Section 1.3. How these factors influence the toxicity of trace elements to inanga will also depend on their physiology (see Section 1.7). The BLM approach is based on data from a few "model" fish species (Paquin et al., 2002). It is not yet known whether such models are applicable to inanga, which exhibit a number of specialized characteristics (e.g. a lack of scales that confers a significant transport function to the skin; Urbina et al., 2012).

Tissue metal accumulation has been shown to relatively accurately predict toxicological consequences of most metals in aquatic biota (Clearwater et al., 2002; Di Toro et al., 2001; Norwood et al., 2003). However, results from laboratory and field often offer contrasting conclusions (Clearwater et al., 2002). Furthermore, metal toxicity has consistently been shown to be species- and habitat-specific (Besser et al., 2007; Dutton and Fisher, 2011; Kraemer et al., 2005b). Therefore, understanding the relationship between water chemistry and metal accumulation in a natural setting is an important step toward predicting metal toxicity and/or calibrating regulatory modelling tools towards non-model organisms. As such, *in situ* field studies are an important approach. Caging is advantageous over field collection as a biomonitoring tool as it allows biological responses, such as

accumulation, to be matched specifically to the local ambient conditions present at a monitoring site (Oikari, 2006). This is especially important in the case of mobile/migratory species like inanga. The aims of the current study were to investigate metal accumulation in inanga as a function of stream water chemistry through the combination of a stream survey and caging study along the West Coast of New Zealand's South Island. The working hypothesis being tested for the stream survey was that fish populations would be negatively correlated with metal contaminated streams. Additionally, caged exposures were expected to lead to trace element accumulation corresponding to dissolved water concentrations.

4.2 Methods

4.2.1 Stream survey

Thirty-four streams along the West Coast of New Zealand were surveyed in February 2013 (Fig. 4.1) for water chemistry and fish presence (Appendix 1). Water quality parameters were determined as per Section 2.5. Surrounding land use was categorized as agricultural, natural/undisturbed, or urban depending on its proximity to farms or towns. In order to be considered agricultural, a farm was present, either surrounding, or within a few meters of the stream. Urban was considered to be within a township, and natural land use was simply the absence of urban or agricultural indicators.

Water and sediment samples were collected and acidified as per Section 2.5 at each of the 34 streams at the time traps were set (see below). Samples were then analyzed *via* ICP-MS as described in Section 2.7.2. For determining whether fish presence was related to stream trace element burden, total and mean concentration factors were calculated for sediment and water as the sum of all trace element concentrations or the mean of trace element concentrations at each site.

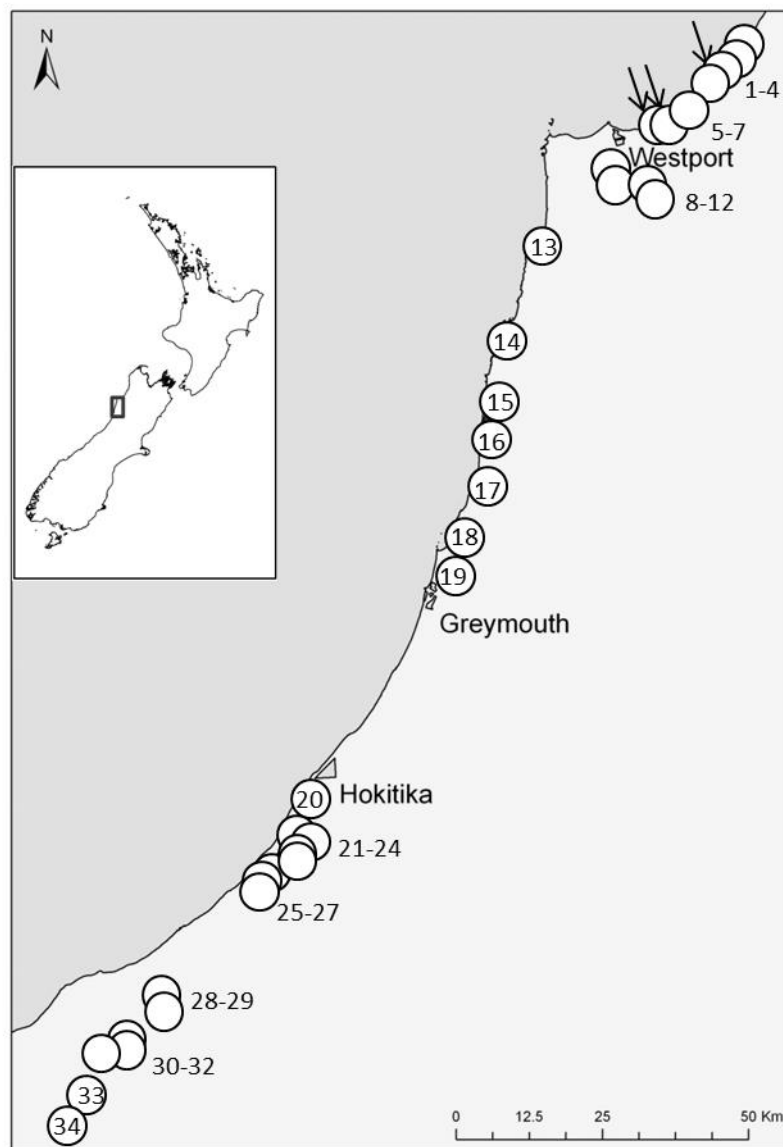


Figure 4.1 Location of sampling points for streams surveyed along New Zealand's West Coast. The source stream located in the town of Westport, and caging study streams (3, 6, and 7) denoted with arrows. Numbers correspond to those in Appendix 2.

4.2.2 Fish survey

In order to estimate fish presence, gee-minnow traps were set in the afternoon, left overnight, and collected the next morning. Fish caught were identified to species level and the number of each recorded before release.

4.2.3 Caging study

Three streams from the survey were selected for a small caging study to determine the effect of water chemistry on metal accumulation. Streams were chosen based on accessibility, fish presence, and total trace metal concentrations. Fairdown No. 1 (FN1) contained elevated Al, Cu, Fe, Ni, and Zn water concentrations; Deadman's Creek (DMC) had moderate contaminant levels; and Jones Creek (JC) was relatively uncontaminated (Table 4.1).

Similar to the stream survey, water chemistry (pH, DO, temperature, and salinity) was measured at each site (Table 4.2). Additionally, water samples were also taken for determination of NOM via methods described in Section 2.6.

Adult inanga were collected, transported, and placed into cages as per Section 2.5. Cages were constructed, tested, and set up as per Section 2.5. Up to ten fish, depending on survival, were collected at both 96 hour and 9 day time intervals at each stream (Table 4.2). Some mortality occurred in DMC and FN1 (Table 4.2), which was a consequence of major storms that occurred between the 96 hour and 9 day sampling periods. After collection, fish were euthanized (as per Section 2.3) and six of the ten fish collected (or approximately 60%) had the gills and gastrointestinal (GI) tract removed for determination of trace element concentrations (hereafter termed incomplete fish). Unfortunately, it was subsequently found that these gill and GI samples had been contaminated and analysis was unable to proceed. The remaining 4 (or 40%) were kept whole. Samples were kept frozen at -20°C before digestion (Section 2.7.1) and determination of metal concentrations (Section 2.7).

Table 4.1 Mean water metal concentrations ($\mu\text{g L}^{-1}$) \pm standard error of mean for source (Oro) and caged streams. Cd and Cr had more than 50% of samples below detection limits and were therefore excluded.

Stream	Abbreviation	n	Al	As	Co	Cu	Fe	Ni	Pb	Zn
Orowaiti	Oro	4	66.4 ± 3.0	1.1 ± 0.01	0.7 ± 0.01	0.8 ± 0.09	$3,092 \pm 121$	0.91 ± 0.01	0.13 ± 0.01	5.4 ± 0.15
Deadman's Creek	DMC	7	167.6 ± 26.8	0.4 ± 0.01	0.4 ± 0.03	0.8 ± 0.10	595 ± 29	0.79 ± 0.07	0.46 ± 0.06	4.8 ± 0.89
Fairdown No. 1	FN1	7	290.8 ± 50.1	1.0 ± 0.04	1.0 ± 0.09	1.6 ± 0.14	649 ± 17	1.27 ± 0.07	0.63 ± 0.07	20.9 ± 1.70
Jones Creek	JC	9	65.8 ± 11.2	0.2 ± 0.01	$0.1 \pm < 0.01$	0.2 ± 0.05	19 ± 3	0.08 ± 0.02	0.05 ± 0.01	1.5 ± 0.86

Table 4.2 Physicochemical parameters and number of fish collected for metal analysis from source stream (Oro) and three caging study streams (DMC, FN1, and JC).

Stream	Salinity (‰)	Dissolved Oxygen (mg L^{-1})	Temperature ($^{\circ}\text{C}$)	pH	Natural Organic Matter (mg C L^{-1})	Number of Fish Sampled at 96 h	Number of Fish Sampled at 9 d
Oro	3.4	11.0	16.5	5.4	4.2	10	10
DMC	0	10.5	12.0	4.9	4.1	9	5
FN1	0	9.9	13.0	5	8.0	10	8
JC	0	10.6	12.5	5.8	2.2	10	10

4.2.4 ICP-MS

Water, sediment, and fish samples were processed and analyzed for Al, As, Cd, Co, Cr, Cu, Fe, Ni, Pb, and Zn *via* ICP-MS as per Section 2.7. Certified reference materials and internal standards were used for quality control as described in Section 2.7.2.

4.2.5 Metal speciation

In order to integrate water chemistry data and predict metal bioavailability, site-specific metal speciation modelling was conducted for all the caging sites. This was performed only for Al, Fe and Cu; the former being the two metals that best exhibited significant differences in metal accumulation between streams, and the latter a metal that remained constant in terms of burden during the caging study. Speciation analysis was performed using MINEQL+ (version 4.5) modelling software (Schecher and McAvoy, 1992), using log K 's of 6.85, 8.89, 2.20, and 17.50 for Cu-NOM, Al-NOM, ferrous iron-NOM and ferric iron-NOM, respectively (Milne et al., 2003). It was assumed that NOM had a metal binding capacity of 480 nmol mg C L⁻¹ (Fujii et al., 2014). The speciation analysis was run twice, once assuming all iron was ferrous (Fe²⁺), a form with low NOM binding affinity, but the dominant iron species in oxic waters; and once assuming the iron was present in a ferric (Fe³⁺) form (high NOM binding affinity, low presence in oxic waters). Free metal ion concentrations were correlated against 9 day combined metal burdens for individual fish, to determine whether ionic metal species and therefore water chemistry, were predictive of accumulation. Correlations were also performed for total metal levels in the water.

4.2.6 Statistical analyses

Both sample types (whole and incomplete) were statistically evaluated separately, but also as a combined sample in order to compare accumulation in whole fish *versus* those without gills and GI tract. These are presented as whole, incomplete, or combined sample groupings.

Statistical testing followed those outlined in Section 2.11 where appropriate. For correlation analysis of survey data, Pearson's product moment correlations were used; and

binomial presence/absence data were evaluated using binomial GLMs. All statistical analyses were conducted using the R software (Version 2.15.0).

4.3 Results

4.3.1 Stream survey

4.3.1.1 Water chemistry

Among the 34 streams surveyed, salinity values ranged from 0.0-0.7‰, pH ranged from 3.9-7.5, dissolved oxygen ranged from 6.5-10.9 mg L⁻¹, and temperature ranged from 14.9-24.4°C (Appendix 1). Granity Creek was the most contaminated stream with the highest water concentrations of Al (29,970.9 µg L⁻¹), Fe (16,757 µg L⁻¹), and Zn (1,288.2 µg L⁻¹); while the remaining 33 streams ranged between 14.9-7,823.2 µg L⁻¹ Al, 3.8-2,179.0 µg L⁻¹ Fe, and 0.18-165.2 µg L⁻¹ Zn (Appendix 2).

4.3.1.2 Sediments

Sediment trace element concentrations were not significantly related to water concentrations. 9 Mile Creek (Kotorepi) had the highest Fe sediment concentrations (115 ± 39 mg kg⁻¹), while Big Eel Creek displayed Co concentrations at least an order of magnitude higher than other streams (348 ± 76 µg kg⁻¹) (Appendix 3). Interestingly, Granity Creek, which had by far the largest concentrations of waterborne Al, Fe, and Zn, had moderate concentrations of these metals in sediments (Appendices 2 & 3).

4.3.1.3 Fish distributions

Fish were caught in twenty-two out of the thirty-four streams surveyed (65%) (Appendix 1). Inanga were caught in six streams, all within approximately 10 km of the sea. The other species caught, in order of highest to lowest abundance were the common bully (*Gobiomorphus cotidianus*), banded kokopu (*Galaxias fasciatus*), longfin eel (*Anguilla dieffenbachii*), koaro (*Galaxias brevipinnus*), giant kokopu (*Galaxias argenteus*), and redfin

bully (*Gobiomorphus huttoni*). Common bullies were the most abundant; having been caught in 26% of streams surveyed. Distributions varied by species, with bullies being found equally distributed in streams with both natural and agricultural land uses. Inanga were most frequently present in streams associated with agricultural land-use (83.3%), and banded kokopu were most frequently found in streams associated with natural land-use (87.5%). Further, inanga and bully distributions were correlated (Pearson's product-moment correlation, $R^2 = 0.868$, $p < 0.01$). Fish presence was not correlated with any of the water quality parameters or related to either total or mean sediment metal concentration factors (binomial GLM, $p > 0.05$; data not shown).

4.3.2 Caging study

4.3.2.1 Stream physicochemical parameters

Physicochemical parameters were relatively similar between the source stream, Orowaiti (Oro) and the three study sites (Table 4.2). The temperature in Oro averaged 16.5°C , and ranged from $12\text{--}13^{\circ}\text{C}$ in the study sites; pH was 5.4 in Oro and ranged from 4.9–5.8 for study sites; and DO averaged 11.0 mg L^{-1} in Oro whereas the study sites ranged from $9.9\text{--}10.6\text{ mg L}^{-1}$. NOM ranged from $2.2\text{--}8.0\text{ mg C L}^{-1}$ in caged streams, with the source (Oro) at 4.2 mg C L^{-1} .

Water metal concentrations varied between caging sites, with some differing by an order of magnitude (Table 4.1). FN1 was the most contaminated stream, with concentrations of all metals (Al, As, Co, Cu, Fe, Ni, Pb, and Zn) being significantly higher than in JC (one-way ANOVA, $p < 0.01$). The source stream (Oro) fell between FN1 and JC for most metals; however, was significantly higher in Fe (mean = $3,104\text{ }\mu\text{g L}^{-1}$) than the caging study streams (range: $18.5\text{--}652.5\text{ }\mu\text{g L}^{-1}$ Fe) (one-way ANOVA, $p < 0.01$). Levels of metals in DMC were usually intermediate; however, water from this stream was significantly lower in As and Co than all other streams (one-way ANOVA, $p < 0.01$). More than 50% of samples had Cd and Cr levels below detection limits and these metals were therefore excluded from statistical analyses.

Sediment samples from JC were generally higher in most metals than other caged streams (Table 4.3). Specifically, JC had significantly higher levels of Al, Cr, and Zn compared

to Oro, DMC, and FN1 (one-way ANOVA, $p < 0.01$). Only As and Cu were not significantly different between stream sediment samples (one-way ANOVA).

4.3.2.2 Metal concentrations in fish

When sample types were combined (both whole and incomplete fish), both Al and Fe accumulation in inanga was significantly higher across caged streams (DMC, FN1, JC) when compared with the source stream (Oro) (Table 4.4, two-way ANOVA, $p < 0.01$). There were no significant differences in As, Cu, Ni, Pb, and Zn accumulation between streams for combined sample types (two-way ANOVA). In terms of exposure period, Al was the only metal to significantly differ between the 96 h and 9 d time points. More than 50% of combined fish samples had Cd, Co, and Cr levels below detection limits and were therefore excluded from statistical analysis.

When whole and incomplete fish sample types were analyzed separately and time points were combined, whole fish from all three caging study streams (JC, DMC, and FN1) had significantly higher Al and Fe accumulation than the unexposed whole fish collected from the source stream (one-way ANOVA, $p < 0.01$) (Table 4.5). Similar to whole fish, incomplete fish samples (whole body minus gill and GI) showed a significantly higher accumulation of Fe in all three caging study streams (JC, DMC, and FN1) than incomplete fish collected at the source stream (time points combined) (two-way ANOVA, $p < 0.01$). However, Al accumulation in incomplete fish was only significantly higher in FN1 than the source (two-way ANOVA, $p < 0.01$). There were no significant differences in the accumulation of As, Cu, Ni, Pb, and Zn for whole or incomplete fish sample types across either exposure period (one-way ANOVA).

When metal accumulation was compared between whole fish and those with their gills and GI tract removed (incomplete fish), there were no significant differences for most metals (As, Fe, Ni, and Zn) (one-way ANOVA). However, there were significant differences in Al, Cu, and Pb accumulation between the two sample types (Fig. 4.2) with all three being significantly higher in whole fish samples in various streams: Al was significantly higher in whole samples than incomplete for DMC and JC (one-way ANOVA, $p \leq 0.01$), Cu was significantly higher in whole samples than incomplete for DMC ($p = 0.026$) and FN1 ($p <$

0.01) (one-way ANOVA), and Pb was significantly higher in whole samples than incomplete in DMC and FN1 (one-way ANOVA, $p = 0.01$ and $p = 0.022$, respectively).

4.3.2.3 Metal speciation

No significant correlations between calculated free metal ions for Al, Fe and Cu and the body burdens of these metals could be determined, irrespective of assumed iron speciation (all relationships negative or with r^2 values ≤ 0.03). Similarly, total water Al, Fe or Cu did not correlate to metal body burdens (all relationships negative or with r^2 values ≤ 0.007).

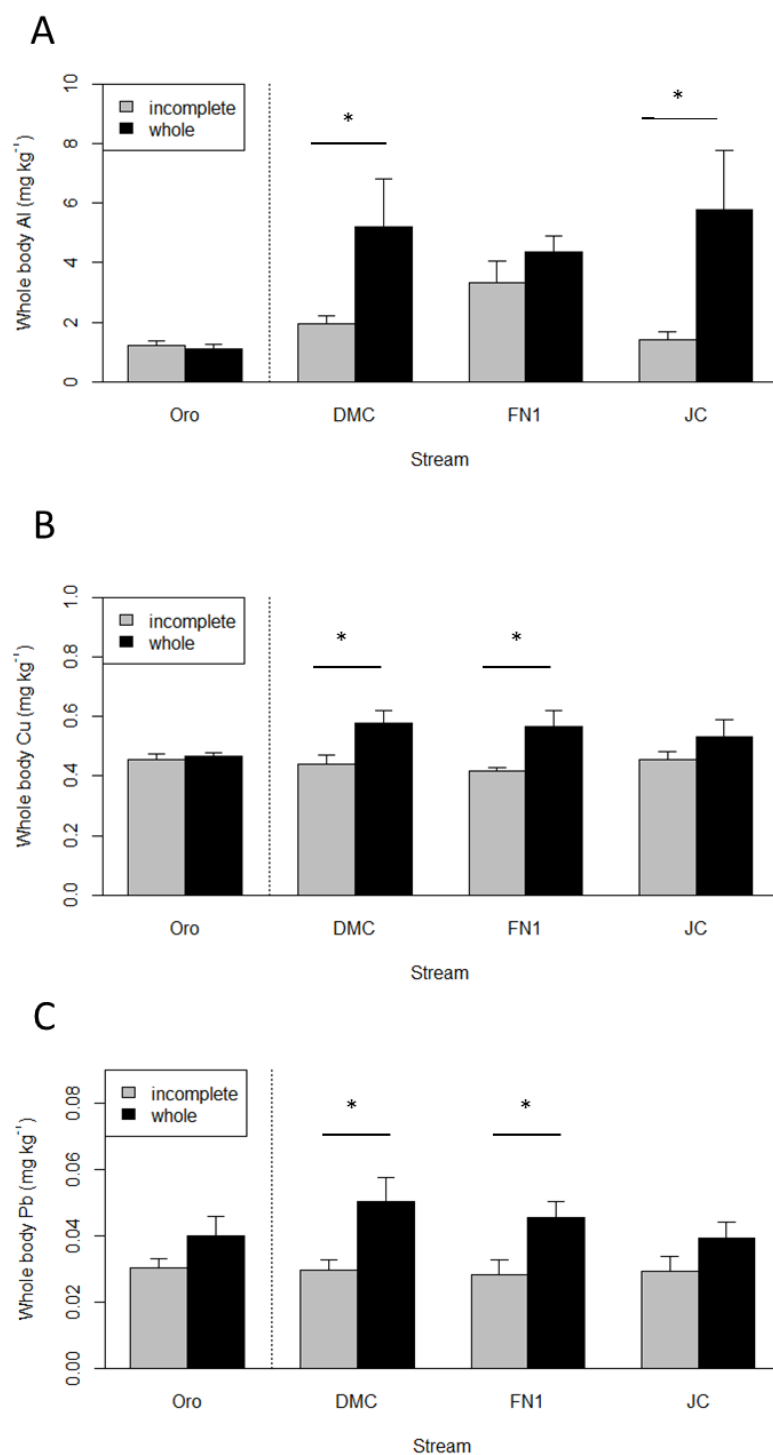


Figure 4.2 A) Al, B) Cu, and C) Pb (mg kg^{-1}) accumulation in whole *versus* incomplete fish sample types in caged (DMC, FN1, and JC) and source (Oro) streams for both sample periods combined (96 h and 9 d). Asterisks denote significant differences between whole and incomplete sample types (one-way ANOVA, $p < 0.05$, n values denoted in Table 4.5).

Table 4.3 Mean sediment metal concentrations (mg kg^{-1}) \pm standard error of mean for source (Oro) and caged streams. Asterisks (*) indicate significant differences in trace element concentrations when compared to Oro. Cd had more than 50% of samples below detection limits and was therefore excluded.

Stream	n	Al ($\times 10^3$)	As	Co	Cr	Cu	Fe ($\times 10^4$)	Ni	Pb	Zn
Oro	1	4.17	2.60	3.71	10.23	5.24	1.16	8.37	10.33	39.33
DMC	2	5.62 ± 0.18	3.40 ± 0.12	3.32 ± 0.08	7.26 ± 0.42	6.70 ± 0.68	0.62 ± 0.05	7.52 ± 0.20	16.05 ± 1.41	42.32 ± 1.13
FN1	2	3.79 ± 0.43	2.21 ± 0.52	1.87 ± 0.10	4.64 ± 0.52	5.40 ± 1.42	2.00 ± 0.08	4.25 ± 0.33	12.34 ± 2.96	23.32 ± 0.67
JC	2	$12.24 \pm 1.03^*$	1.66 ± 0.03	8.93 ± 1.10	$40.47 \pm 1.32^*$	7.88 ± 0.87	1.10 ± 0.18	19.30 ± 2.64	15.12 ± 0.35	$70.88 \pm 4.36^*$

Table 4.4 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for combined fish sample types (whole and incomplete fish) (combined 96 hour and 9 day exposure periods). Asterisks (*) indicate significant differences ($p < 0.05$) from the source stream (Oro). Cd, Co, and Cr had more than 50% of samples below detection limits and were therefore excluded.

Stream	n	Al	As	Cu	Fe	Ni	Pb	Zn
Oro	20	1.16 ± 0.12	0.61 ± 0.05	0.46 ± 0.01	14.16 ± 0.64	0.05 ± 0.01	0.03 ± 0.003	56.04 ± 1.95
DMC	14	$2.88 \pm 0.61^*$	0.57 ± 0.03	0.48 ± 0.03	$24.25 \pm 2.74^*$	0.08 ± 0.02	0.04 ± 0.004	57.23 ± 3.33
FN1	18	$3.74 \pm 0.49^*$	0.62 ± 0.04	0.48 ± 0.03	$21.25 \pm 1.83^*$	0.06 ± 0.01	0.03 ± 0.004	57.83 ± 2.41
JC	19	$3.03 \pm 0.87^*$	0.67 ± 0.04	0.48 ± 0.03	$19.14 \pm 1.10^*$	0.04 ± 0.01	0.03 ± 0.003	61.05 ± 3.17

Table 4.5 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for whole and incomplete fish samples (combined 96 hour and 9 day exposure periods). Asterisks (*) indicate significant differences ($p < 0.05$) between fish accumulation in caged streams *versus* the source stream (Oro). Cd, Co, and Cr had more than 50% of samples below detection limits and were therefore excluded.

Stream	Fish	n	Al	As	Cu	Fe	Ni	Pb	Zn
Oro	incomplete	12	1.2 ± 0.17	0.7 ± 0.04	0.5 ± 0.02	13.7 ± 0.7	0.05 ± 0.01	0.03 ± 0.003	55.7 ± 1.90
	whole	8	1.1 ± 0.15	0.5 ± 0.09	0.5 ± 0.01	14.8 ± 1.2	0.05 ± 0.01	0.04 ± 0.01	56.5 ± 4.15
DMC	incomplete	10	1.9 ± 0.27	0.6 ± 0.04	0.4 ± 0.03	$20.2 \pm 1.0^*$	0.08 ± 0.03	0.03 ± 0.003	58.9 ± 4.38
	whole	4	$5.2 \pm 1.61^*$	0.6 ± 0.08	0.6 ± 0.04	$34.3 \pm 7.8^*$	0.07 ± 0.01	0.05 ± 0.01	53.0 ± 4.02
FN1	incomplete	11	$3.3 \pm 0.72^*$	0.6 ± 0.05	0.4 ± 0.01	$20.1 \pm 2.7^*$	0.06 ± 0.01	0.03 ± 0.004	56.8 ± 1.75
	whole	7	$4.4 \pm 0.52^*$	0.7 ± 0.07	0.6 ± 0.05	$23 \pm 2.2^*$	0.06 ± 0.01	0.05 ± 0.01	59.4 ± 5.79
JC	incomplete	12	1.4 ± 0.24	0.7 ± 0.06	0.5 ± 0.03	$17.3 \pm 1.1^*$	0.05 ± 0.01	0.03 ± 0.004	59.5 ± 4.28
	whole	7	$5.8 \pm 1.98^*$	0.7 ± 0.03	0.5 ± 0.06	$22.3 \pm 1.8^*$	0.04 ± 0.01	0.04 ± 0.01	63.8 ± 4.69

4.4 Discussion

4.4.1 Stream survey

Fish presence was not significantly related to either total or mean trace element water concentration factors across streams, suggesting the presence of inanga was not influenced by total stream metal burdens. Although some fish species show a strong behavioral avoidance of metals (Atchison et al., 1987); the banded kokopu (*Galaxias fasciatus*), a fish closely related to inanga, does not avoid Cd (Baker and Montgomery, 2001), the only metal for which avoidance data exists in galaxiid fish. This lack of avoidance may be one factor explaining the lack of relationship between fish occurrence and water metals. That inanga were not specifically excluded from streams with elevated water metal levels also indicates that inanga are relatively resilient to any toxic effects of metals, which might otherwise exclude them from contaminated streams.

The stream survey also alluded to general habitat preferences for inanga and a few other species of native New Zealand fish. Inanga appeared to prefer streams surrounded by agricultural land use, banded kokopu were found more commonly in natural streams, and bullies were more flexible, with relatively equal distributions across land uses. Further, inanga and bully distributions were found to be correlated with one another supporting species-associations found in previous studies (Jowett and Richardson, 1996; Minns, 1990).

4.4.2 Caging study

This study showed that over the course of a 9 day caged exposure, inanga trace element body burdens remained relatively unchanged. The only metals that significantly changed as a result of transplantation were Al and Fe. Trace element accumulation in fish did not correspond with sediment or water (either total or free ion) concentrations in this study. Although modelling approaches that predict toxicity from free metal ions are the basis of current regulatory tools (e.g. BLM; Paquin et al., 2002), predictions of bioavailability based on total or dissolved metal concentrations have been less successful (Janssen et al., 2003). This is consistent with the results of the current study.

The lack of significant correlations between trace element body burdens and water and sediment trace elements suggest that patterns of Al and Fe accumulation, in particular, are shaped by alternative sources. Dietary uptake is one likely factor. Inanga are generalist feeders and their diet consists of crustaceans, insects, detritus, and other opportunistic prey (Becker and Laurenson, 2007). A previous investigation in West Coast streams showed that different potential inanga prey species varied significantly in terms of their Al and Fe burdens, even within taxa (Winterbourn et al., 2000). Although neither of these metals is known to biomagnify through food chains (Oberholster et al., 2012; Winterbourn et al., 2000), depending on the prey species available to caged fish, different levels of dietary exposure to these metals may have occurred. Such a phenomenon may be exacerbated by the fact that metal-contaminated streams display reduced macroinvertebrate diversity (e.g. Doi et al., 2007), and that those species with greatest tolerance are usually those that can withstand higher metal burdens (e.g. Kalantzi et al., 2014). Thus it is possible that the higher burdens of Al and Fe in inanga from some of the caging streams may represent the subsistence on a restricted diet with elevated metal tissue levels.

The one metal where waterborne accumulation may have played an important role is Al. Al has been shown to increase the production of branchial mucus (Wood et al., 1988); and acidic waters, such as those in the current study, promote precipitation of Al onto this mucus layer (e.g. Winter et al., 2005). This results in toxicity through the impairment of gill-based respiratory and ionoregulatory functions (Gensemer and Playle, 1999). In the present study, the higher levels of Al in whole fish (with gills intact) relative to incomplete fish (with gills removed) may reflect the presence of branchial Al precipitation.

Inanga with gills and GI tracts removed also showed significant differences to whole fish in terms of Cu and Pb accumulation for DMC and FN1 (Fig. 4.2). Studies have shown that Pb behaves similarly to Al, with a 10 day exposure leading to high concentrations in the gill and intestine of fish, but little found in other tissues (Tao et al., 1999). This is consistent with the findings of the current study with respect to changes in accumulation of caged fish relative to control (Oro) fish. It might therefore be anticipated that these non-essential elements such as Al and Pb are trapped in epithelial tissues by mechanisms that limit their entry, whereas essential elements such as Zn, are absorbed readily through dedicated uptake pathways (Bury et al., 2003). However, while this pattern holds for Al/Pb (non-essential, not internalized) and Zn/Fe (essential and thus taken up for utilization), it does not

for Cu (accumulates in epithelia instead of being absorbed into internal tissues) or for Cr (a toxic element that might be expected to be limited in terms of uptake). The reasons underlying the accumulation patterns observed are likely to be complex. Factors such as metal concentration (essential elements are toxic above a certain threshold), affinity of metal for binding gut or branchial mucus, gut chemistry and its effect on metal speciation, and the availability of transport systems will all likely play a role in shaping the accumulation patterns observed (Bury et al., 2003; Ojo and Wood, 2007; Wood, 2012). It is worth noting, though, that an *in vitro* study examining the transport of a variety of metals across rainbow trout intestine also showed that uptake rates did not correspond to metal essentiality (Ojo and Wood, 2007), a finding that corresponds with those shown here.

Interestingly, water samples from the source stream (Oro) had nearly 5 times the total Fe concentration of the next highest stream (FN1) (Table 4.1). As such, a depuration of Fe might have been expected following transfer to caging streams. However, fish in all caged streams accumulated more Fe than fish collected directly from the source stream. One possible explanation for this involves mechanisms of Fe uptake. Absorption of Fe is primarily achieved by the DMT1, which is a proton symporter (Bury et al., 2003). Consequently at lower pH's higher Fe uptake could be expected (at least through gill-based transport). This might explain the enhanced uptake in DMC and FN1, as these streams both had water pH values approximately 0.5 units lower than the source stream. However, JC had a higher pH, but still resulted in fish with elevated Fe burdens relative to Oro, and as mentioned previously total water Fe and speciated free Fe did not correlate with uptake. This implies Fe accumulation in streams with lower Fe concentrations may have been related to changes in gastrointestinal uptake.

In Fe-deficient rats, an increase in Fe supply leads to a rapid reduction of DMT1 transporter expression (Arita et al., 2010). Conversely in Fe-sufficient animals, a reduction in Fe availability causes a rapid increase in DMT1 expression (Canonne-Hergaux et al., 1999). It is reasonable to assume that fish from Oro were Fe-sufficient given the elevated Fe levels in their habitat. This is especially likely to be true given that these fish were also exposed to waters of higher salinity, which may have facilitated the use of the gut as an uptake route. In this study, a single spot sample recorded a salinity of 3.4‰, but as a coastal lagoon, higher salinities will occur at the Oro site with tidal changes. Freshwater inanga, like other teleost fish, are faced with diffusive ion loss and water influx (Urbina et al., 2013). However,

at salinities above their isosmotic point (10-15‰), fish must deal with diffusive ion influx and diffusive water loss. In order to compensate for this they drink. This will result in elevated Fe levels coming in contact with the gastrointestinal epithelium, facilitating Fe uptake via DMT1 transporters in the gut (Kwong et al., 2010). When these Fe-sufficient fish were placed in environments of lower Fe, an upregulation of DMT1 would be expected, resulting in an increase in Fe uptake, and leading to the higher body burdens observed in these fish, despite the lower environmental Fe levels measured.

Overall, inanga were found to significantly accumulate only Al and Fe in caged streams, suggesting a strong regulation of other trace elements. Speciation analysis showed no correlations of free ion concentrations to Al or Fe accumulation. This suggests predicting accumulation based on this free ion models would not be applicable for inanga. It is possible that the source for this additional Al and Fe was through the diet. As inanga were found in a range of contaminated streams, they do not appear to purposefully avoid contaminants, as such, it is likely that inanga may be more tolerant to trace element toxicity.

Chapter 5: Assessing trace element toxicity in inanga, *Galaxias maculatus*, using biomarkers after an *in situ* caging study along the West Coast of New Zealand's South Island

5.1 Introduction

The results of Chapter 4 showed that, in general, short-term changes in body burden did not correlate to environmental levels of trace elements. Accumulation is an important endpoint of exposure, as it is a good predictor of toxicity (MacRae et al., 1999). However, the relationship between accumulation and toxicity varies between fish species, and depends on a number of factors including mechanism of toxic effect, physiology, and previous exposure history (Besser et al., 2007; Paquin et al., 2002). As such, a broader *in situ* field study was necessary to understand short-term accumulation and its subsequent toxic effects in inanga; a species for which almost nothing is known regarding mechanisms of trace element toxicity. The current chapter investigated trace element accumulation in inanga caged in six streams along the West Coast, three of which were also included in the first study (Chapter 4). However, in this study, fish gills and liver were also removed for biomarker analysis to identify toxic endpoints that resulted from caged exposure.

In situ caging studies have been applied as ecological monitoring tools for multiple environmental pollutants (Blom et al., 1998; Cazenave et al., 2014; Harries et al., 1997; Klobucar et al., 2010; Kraemer et al., 2005a). After exposures, accumulation and/or biomarkers are often used to quantify site-specific contamination (Cazenave et al., 2014; Harries et al., 1997; Reynders et al., 2008). In fact, Cazenave et al. (2014) found oxidative stress measures (GST, GR, TBARS, and CAT) and metabolic parameters were key biomarkers in caged *Prochilodus lineatus* exposed to wastewater. Determining the relationships between trace element accumulation and toxicity for inanga in their natural habitats is an important step in understanding how metals affect this species. Such data allows better predictions to be made regarding potential impacts, leading to a better understanding of how to protect habitats.

Accumulation has traditionally been used to predict toxic consequences of metal exposures (Clearwater et al., 2002; Di Toro et al., 2001; Norwood et al., 2003). Short-term changes in whole body and/or organ accumulation have been shown to serve as an effective indicator of exposure. Using this relationship between accumulation and toxicity allows models like the BLM to predict toxic effects. Biomarkers are one tool that can be used to provide specific indicators of more subtle, sub-lethal toxic effects. By determining sub-lethal effects, responses to contaminant exposure can be measured before lethal levels are

reached, which is important in a regulatory context. Some of these biomarkers can also allow for contaminant monitoring without lethal sampling. For example, gill NKA can be measured with just a small subsection of the gill (in large fish), preventing lethal consequences (McCormick, 1993).

The gill and liver of freshwater fish often display the largest changes in biochemical biomarkers (Bagnyukova et al., 2006; Craig et al., 2007; Loro et al., 2012). This is likely due to increased exposure, as the gills are in constant contact with contaminated water and the liver filters/stores absorbed trace elements (Di Toro et al., 2001). As such, these tissues are commonly used to measure biomarker responses. For example, the synthesis of proteins capable of binding and storing metals (i.e. metallothionein) in the gill can function to sequester and detoxify metals at low concentrations. This sequestration, however, is limited in terms of exposure length and concentration (Chowdhury et al., 2005; Kraemer et al., 2005a). Measures of oxidative stress have often been used as indicators for metal toxicity in both gill and liver tissues (van der Oost et al., 2003). Oxidative stress is a common response in fish to metal contaminants (see Section 1.5). Biomarkers such as CAT, GST, SOD, and LPO measures have all been previously used in trace element exposure studies (Loro et al., 2012; Pandey et al., 2008). The current study used LPO as a biomarker to determine sub-lethal effects of trace element exposure in inanga. The streams in this chapter had levels of Cu, Fe, Ni, Pb, and Zn shown to cause LPO damage in other fish (Bagnyukova et al., 2006; Firat et al., 2011; Loro et al., 2012; Lushchak, 2011), so TBARS in the liver was measured in order to determine resulting lipid peroxidation.

Gills were used for the determination of NKA activity as its activity is intricately linked to ion balance within the cell (Hwang and Lee, 2007). NKA has previously been shown to change in response to trace element exposure (see Section 1.5) (Fernandes et al., 2013; Griffitt et al., 2007; Hoyle et al., 2007). For example, gill NKA activity was shown to significantly decrease as a result of Cu exposure in fish (Craig et al., 2010). As a range of trace elements were found elevated in West Coast streams (Chapter 4), this biomarker was also measured in the gills of caged fish.

The aim of this study was to evaluate the *in situ* changes in trace element accumulation in inanga across a range of contaminated streams. Biomarkers of TBARS and NKA were used to determine specific biochemical responses to metal exposure in order to better understand mechanisms of toxicity. This mechanistic understanding can then be used

to establish a relationship between short-term metal exposure, tissue metal accumulation, and toxicity. The working hypothesis being tested was that caged exposures would lead to trace element accumulation corresponding to dissolved water concentrations.

5.2 Methods

5.2.1 Study sites

Six streams along the West Coast of New Zealand were chosen based on known water chemistry and metal contaminant levels (Chapter 4). Three sites were north of Westport: Deadman's Creek (DMC), Fairdown No. 1 (FN1), and Jones Creek (JC); while three were located to the south: Limestone Creek (LC), Maher's Creek (MC), and Coal Creek No. 2 (CC) (Fig. 5.1). Sites varied in salinity, pH, temperature, DO, NOM and metal contaminant loads (Table 5.2). LC and MC were not close to urban areas or agriculture, and were therefore classified as 'natural.' The other four streams (FN1, DMC, CC, and JC), however, were in close proximity to farms. JC and MC were the two 'clean' streams, with relatively low contaminant levels. FN1 and DMC were both relatively high in Fe, Pb, Cu, and Zn; and LC and CC had moderate concentrations of most metals.

Water quality parameters were measured at each site (pH, temperature ($^{\circ}\text{C}$), salinity (‰), and NOM (mg C L^{-1}), see Sections 2.5 & 2.6); and surrounding land use was noted (Table 5.1).

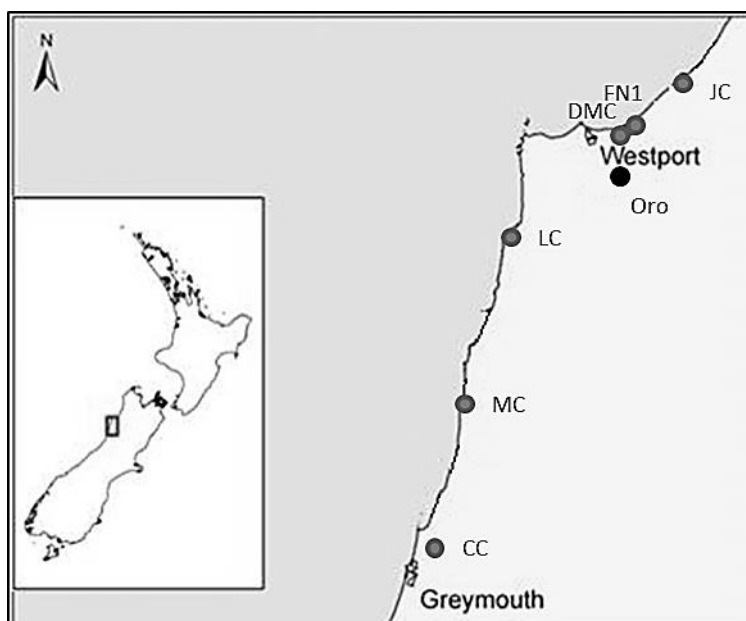


Figure 5.1 Location of streams along New Zealand's West Coast: source stream denoted in black, caged streams in grey.

5.2.2 Fish collection

Adult inanga (*Galaxias maculatus*) were collected from the Orowaiti Lagoon located just outside of Westport (3.4 ‰ salinity, 11.01 mg L⁻¹ dissolved oxygen, 16.5°C, pH 5.6, and 4.2 mg C L⁻¹ NOM) and transported to caged streams using methods described in Section 2.5. Five fish were removed, euthanized, and dissected, in the same manner (Section 2.5), from the source stream as a reference for caged streams (Table 5.1).

5.2.3 Caged exposures

Twenty fish (weight range: 0.2-4.8 g, mean: 1.3 g) were placed in each cage as described in Section 2.5. Sediments and both filtered (for dissolved trace elements) and unfiltered (for total trace elements) water samples were collected from each stream. Water and sediment samples were treated as described in Section 2.5. The number of sediment samples was uneven as some were contaminated at an unknown stage of processing, and were subsequently excluded from analysis. Only one 'time 0' sample of fish was collected from the source stream in order to compare 96 h and 10 d exposures to a singular initial

mean value. Caged fish were collected at two time points in order to determine changes in accumulation over time.

Up to ten fish, depending on survival, were collected at both 96 hour and 10 day time intervals at each stream. Fish were euthanized as described in Section 2.3 before gill and liver samples were removed, flash frozen in liquid nitrogen, and kept at -80°C before biomarker analysis. The remainder of the body was kept frozen at -20°C before determination of metal concentrations.

5.2.4 Analytical chemistry and biomarker analyses

Fish samples were digested and analyzed by ICP-MS for the determination of Al, As, Co, Cd, Cr, Cu, Fe, Ni, Pb, and Zn as described in Section 2.7.2. Additionally, LPO in the liver and NKA activity in the gills were measured as described in Sections 2.8.1 and 2.8.2, respectively.

5.2.5 Statistical analyses

Data were analyzed as described in Section 2.11. The Pearson's product-moment correlation was used for correlation analysis of environmental variables: total water, dissolved water, and sediment trace element concentrations, with r^2 and p-values provided. Eight different parameters were tested for correlations (*via* Pearson's product-moment) with TBARS. These comprised of mean total water, mean dissolved water, and mean sediment trace element concentrations; sum total of oxygen reactive trace elements (Cr, Cu, and Fe; as per Valko et al., 2005) for total water, dissolved water, and sediments; stream pH and NOM concentration. All statistical analyses were conducted using the R program (Version 2.15.0) with $p < 0.05$ considered significant.

5.3 Results

5.3.1 Fish mortality

Before the first collection, and after three days of exposure, four fish died at LC; as such, only eight of the remaining sixteen were collected at 96 h. Four fish were also found dead in DMC during the first collection (96 h); and with only 14 fish counted, seven were collected. It is likely that the missing two fish escaped as a result of flawed trap closures. Fish also escaped from the enclosure in JC as only 17 fish were counted at 96 h and none appeared to have died; as such, only eight were collected. MC also had three fish die before the first collection, so only eight were collected. There was a very large storm on Day 5 that resulted in rapid increases in water flow and caused cages to lose anchor. As a result of the storm, both CC and MC lost the remainder of their fish. The storm also caused four fish deaths in LC, leaving only three alive (one was lost), one dead in DMC (two were lost), and two dead in JC (one was lost). As deaths did not relate to contamination levels, it is unlikely that toxicity was the cause of death.

5.3.2 Water chemistry

Physicochemical parameters varied between the source stream (Oro) and the six study sites (Table 5.1). NOM ranged between 0.8-8.0 mg C L⁻¹, pH ranged from 5.6-6.9, and temperature ranged from 12.3-16.5°C across streams. Total water Al concentrations ranged from 45-291 µg L⁻¹ with the highest concentrations in LC and FN1 and the lowest in MC and CC (Table 5.2), with total Al water concentrations not significantly correlated to water pH (Pearson's product-moment correlation). Dissolved water Al concentrations ranged between 47-165 µg L⁻¹ (Table 5.2). Total water Fe concentrations were significantly higher in the source stream (Oro) ($2,471 \pm 452$ µg L⁻¹) than caged streams (59-769 µg L⁻¹) (one-way ANOVA, $p < 0.01$). With respect to dissolved water Fe concentrations, FN1 had the highest measured value (623 µg L⁻¹), contrasting with the results for total Fe levels. Total As, Cr, Cu and Zn were highest in the source stream (Oro), while total Ni was highest in MC (Table 5.2). The dissolved water sample from MC was contaminated and was therefore excluded from the results (Table 5.2).

Table 5.1 Physicochemical parameters and fish n values for source (Oro) and caging study streams.

Abbreviation	Stream	Land Use	Dissolved Oxygen (mg L ⁻¹)	Salinity (‰)	Temperature (Celsius)	pH	Natural Organic Matter (NOM) (mg C L ⁻¹)	Number of Fish Sampled at 96 h	Number of Fish Sampled at 10 d
Oro	Orowaiti	natural	11.0	3.4	16.5	5.6	4.2	5	
CC	Coal Creek No. 2	agricultural	9.4	0.0	16.5	6.1	3.4	10	--
DMC	Deadman's Creek	agricultural	9.5	0.0	15.6	6.0	4.1	7	4
FN1	Fairdown No. 1	agricultural	9.2	0.0	15.6	5.9	8.0	10	10
JC	Jones Creek	agricultural	10.2	0.1	15.0	6.4	2.2	8	6
LC	Limestone Creek	natural	10.9	0.1	13.6	6.9	1.5	8	3
MC	Maher's Creek	natural	9.3	0.1	14.2	6.5	0.8	8	--

Table 5.2 Mean total and dissolved water trace element concentrations ($\mu\text{g L}^{-1}$) for source (Oro) and caged streams. Cd, Co, and Pb had more than 50% of samples below detection limits and were therefore excluded. ANZECC trigger values ($\mu\text{g L}^{-1}$) for protection of 95% of freshwater biota are included for reference (ANZECC/ARMCANZ, 2000).

Stream	sample type	n	Al	As	Cr	Cu	Fe	Ni	Zn
Oro	dissolved	1	124	0.5	0.3	0.8	524	0.6	1.5
	total	5	199 \pm 108	1.2 \pm 0.3	3.5 \pm 2.3	7.1 \pm 6.20	2,471 \pm 452	1.1 \pm 0.30	25.5 \pm 20.9
CC	dissolved	2	67 \pm 18	0.2 \pm 0.03	0.2 \pm 0.03	0.2 \pm 0.001	259 \pm 34	0.7 \pm 0.003	1.2 \pm 0.2
	total	2	101 \pm 25	0.2 \pm 0.02	0.2 \pm 0.002	0.3 \pm 0.03	397 \pm 47	0.7 \pm 0.03	1.9 \pm 0.2
DMC	dissolved	3	101 \pm 8	0.3 \pm 0.01	0.2 \pm 0.02	0.4 \pm 0.01	360 \pm 29	0.5 \pm 0.01	2.7 \pm 0.1
	total	10	160 \pm 19	0.4 \pm 0.01	0.1 \pm 0.02	0.7 \pm 0.10	594 \pm 22	0.7 \pm 0.10	4.4 \pm 0.6
FN1	dissolved	3	165 \pm 15	1.0 \pm 0.10	0.3 \pm 0.02	1.0 \pm 0.10	623 \pm 25	0.8 \pm 0.03	9.7 \pm 0.5
	total	10	272 \pm 36	1.1 \pm 0.10	0.2 \pm 0.06	1.5 \pm 0.10	769 \pm 65	1.1 \pm 0.10	17.8 \pm 2.0
JC	dissolved	3	47 \pm 8	0.3 \pm 0.02	0.1 \pm 0.01	0.1 \pm 0.01	9 \pm 3	0.1 \pm 0.01	0.6 \pm 0.1
	total	12	126 \pm 65	0.2 \pm 0.01	0.1 \pm 0.02	0.2 \pm 0.04	59 \pm 42	0.1 \pm 0.03	1.6 \pm 0.6
LC	dissolved	3	124 \pm 13	0.3 \pm 0.03	0.1 \pm 0.01	0.3 \pm 0.20	184 \pm 57	1.0 \pm 0.10	1.8 \pm 0.3
	total	3	291 \pm 36	0.6 \pm 0.04	0.2 \pm 0.02	0.2 \pm 0.03	641 \pm 68	1.1 \pm 0.10	4.0 \pm 0.4
MC	total	1	45	0.3	2.1	0.8	134	2.8	0.8
ANZECC Trigger Value			0.8 at pH < 6.5 5.5 at pH > 6.5	As(III) 24.0 As(V) 4.5	Cr(III) 3.3 Cr(VI) 1.0	1.4 ¹	300 ²	None available	8 ¹

¹applies to water hardness > 30 mg L⁻¹ CaCO₃, ²ANZECC found insufficient data to calculate, so the Canadian value was used instead.

Sediment concentrations of all trace elements (Al, As, Co, Cr, Cu, Fe, Ni, Pb, and Zn) were highest in the source stream (Oro) (Table 5.3). CC and FN1 had the lowest concentrations of Al, Co, Cr, Fe, Ni, and Zn in sediments. Most sediment trace element concentrations (Al, As, Co, Cr, Cu, Fe, Pb, and Zn) were not correlated to total water trace element concentrations (Pearson's product-moment); however, sediment Ni concentrations were positively correlated to total Ni water concentrations (Pearson's product-moment, $p = 0.03$, $r^2 = 0.80$). The relationship between sediment trace element concentrations and dissolved trace element concentrations showed no correlations, aside from dissolved Ni concentrations (Pearson's product-moment, $p < 0.01$, $r^2 = 0.90$).

5.3.3 Metal accumulation in fish

Al was the only metal to significantly change in terms of accumulated burden across streams when compared to the source (Table 5.4; two-way ANOVA, $p = 0.01$). Fish in both LC and MC accumulated significantly more Al (Fig 5.2; one-way ANOVA $p = 0.039$ and $p < 0.01$, respectively) than Oro. Fish Fe accumulation in LC and MC was higher than those from the source, but fell just below significance (one-way ANOVA $p = 0.063$ and $p = 0.054$, respectively). No other trace elements (As, Cr, Cu, Ni, and Zn) were found to significantly differ in accumulation between caging streams and the source stream (Table 5.4, two-way ANOVA). No trace elements (Al, As, Cr, Cu, Fe, Ni, and Zn) showed significant differences in accumulation between the 96 h and 9 d time points (one-way ANOVA).

Table 5.3 Mean sediment metal concentrations (mg kg^{-1}) for source (Oro) and caged streams; n values were based on separate collection times. Cd had more than 50% of samples below detection limits and was therefore excluded.

Stream	n	Al ($\times 10^3$)	As	Co	Cr	Cu	Fe ($\times 10^3$)	Ni	Pb	Zn
Oro	2	14.8 \pm 11	11.3 \pm 8.7	9.8 \pm 6.1	26 \pm 16	17 \pm 12	33.8 \pm 23	22 \pm 14	23 \pm 13	93 \pm 53
CC	1	0.9	1.8	1.2	2	0.9	4.8	3	3	12
DMC	4	3.5 \pm 1	2.5 \pm 0.6	2.5 \pm 0.6	5 \pm 1	4.5 \pm 1.3	7.9 \pm 2	5 \pm 1.4	12 \pm 2.6	29 \pm 8
FN1	4	2.4 \pm 1	1.5 \pm 0.5	1.2 \pm 0.4	3 \pm 1	3.3 \pm 1.3	4.2 \pm 1	3 \pm 0.8	9 \pm 2.3	16 \pm 4
JC	4	7.4 \pm 3	1.2 \pm 0.3	5.9 \pm 1.8	27 \pm 8	5.2 \pm 1.6	13.1 \pm 4	13 \pm 4	11 \pm 2.6	47 \pm 14
LC	2	2.9 \pm 0.5	6.7 \pm 1.2	3.6 \pm 0.5	9 \pm 1	2.5 \pm 0.2	10.4 \pm 2	7 \pm 1	4 \pm 0.1	24 \pm 3
MC	1	4.2	8	9.2	10	10.6	16.5	18	12	44

Table 5.4 Mean fish metal concentrations (mg kg^{-1}) \pm standard error of mean for 96 hour and 10 day exposure periods. Asterisk (*) indicates significant differences from the source (Oro). Cd, Co, Cr, and Pb had more than 50% of samples below detection limits and were therefore excluded.

Stream	Time	n	Al	As	Cu	Fe	Ni	Zn
Oro	source	5	0.43 ± 0.07	0.316 ± 0.05	0.302 ± 0.06	8.0 ± 1.7	0.023 ± 0.01	18.9 ± 3.3
CC	96 h	10	2.97 ± 0.95	0.437 ± 0.05	0.345 ± 0.02	19.2 ± 4.1	0.031 ± 0.01	28.6 ± 4.0
DMC	96 h	7	1.60 ± 0.39	0.423 ± 0.05	0.402 ± 0.04	16.2 ± 3.0	0.024 ± 0.01	24.4 ± 2.0
	10 d	4	1.96 ± 0.62	0.536 ± 0.11	0.359 ± 0.04	15.2 ± 3.5	0.032 ± 0.01	27.7 ± 3.1
FN1	96 h	10	1.34 ± 0.25	0.457 ± 0.06	0.353 ± 0.02	12.0 ± 1.2	0.024 ± 0.004	24.0 ± 1.2
	10 d	10	3.29 ± 0.54	0.452 ± 0.04	0.451 ± 0.11	21.5 ± 2.9	0.028 ± 0.003	26.3 ± 1.9
JC	96 h	8	1.80 ± 0.41	0.514 ± 0.06	0.402 ± 0.03	11.1 ± 1.1	0.027 ± 0.003	27.9 ± 3.0
	10 d	6	2.57 ± 0.60	0.381 ± 0.07	0.346 ± 0.02	12.1 ± 1.2	0.024 ± 0.004	26.2 ± 3.3
LC	96 h	8	$4.90 \pm 2.1^*$	0.341 ± 0.05	0.323 ± 0.02	21.3 ± 5.8	0.049 ± 0.01	28.9 ± 5.5
	10 d	3	$4.31 \pm 1.4^*$	0.271 ± 0.07	0.333 ± 0.03	16.2 ± 3.9	0.039 ± 0.01	21.4 ± 2.5
MC	96 h	8	$5.03 \pm 1.5^*$	0.387 ± 0.06	0.395 ± 0.03	19.2 ± 3.6	0.044 ± 0.01	27.7 ± 2.6

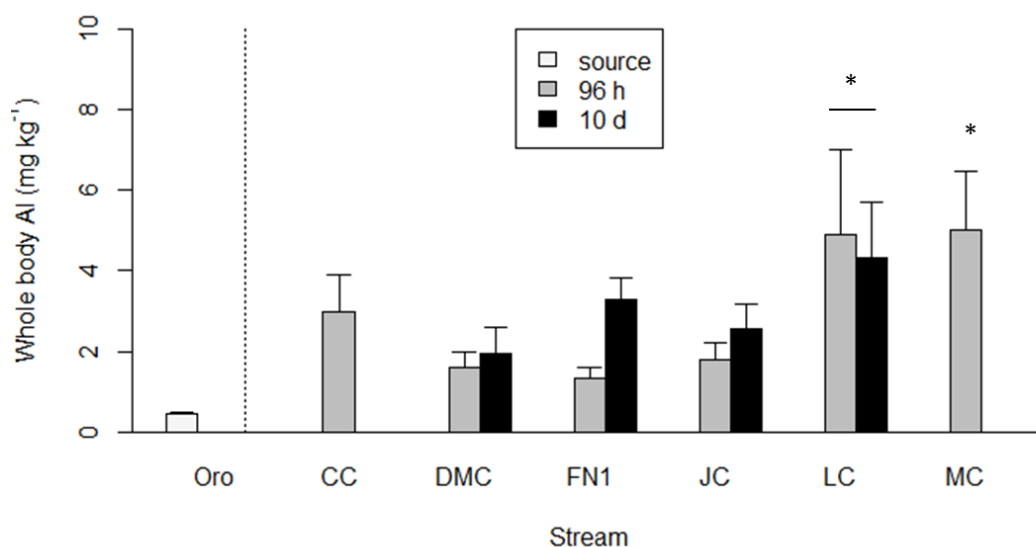


Figure 5.2 Fish Al accumulation (mg kg^{-1}) across source and caged streams (n-values in Table 5.4). A single asterisk (*) denotes significant difference from the source, while an asterisk with bar (—*) denotes an overall site difference from the source (two-way ANOVA).

Whole body Ni accumulation showed significant positive correlations to Ni concentrations in all environmental measures: total water ($p < 0.01$, $r^2 = 0.10$), dissolved water ($p = 0.03$, $r^2 = 0.06$), and sediment ($p = 0.02$, $r^2 = 0.06$) (Fig. 5.3; Pearson's product-moment correlation). Al accumulation was only significantly correlated with total water Al concentrations (Pearson's product-moment correlation, $p < 0.01$, $r^2 = 0.31$). Al body burdens were also significantly correlated with pH (Fig. 5.4; Pearson's product-moment correlation, $p < 0.01$, $r^2 = 0.13$) and NOM levels in streams (Pearson's product-moment correlation, $p = 0.04$, $r^2 = 0.05$). Although these correlations were significant, none of them showed strong relationships, with 0.31 being the largest r^2 value calculated. No other trace element accumulation was positively correlated to their corresponding sediment or water concentrations.

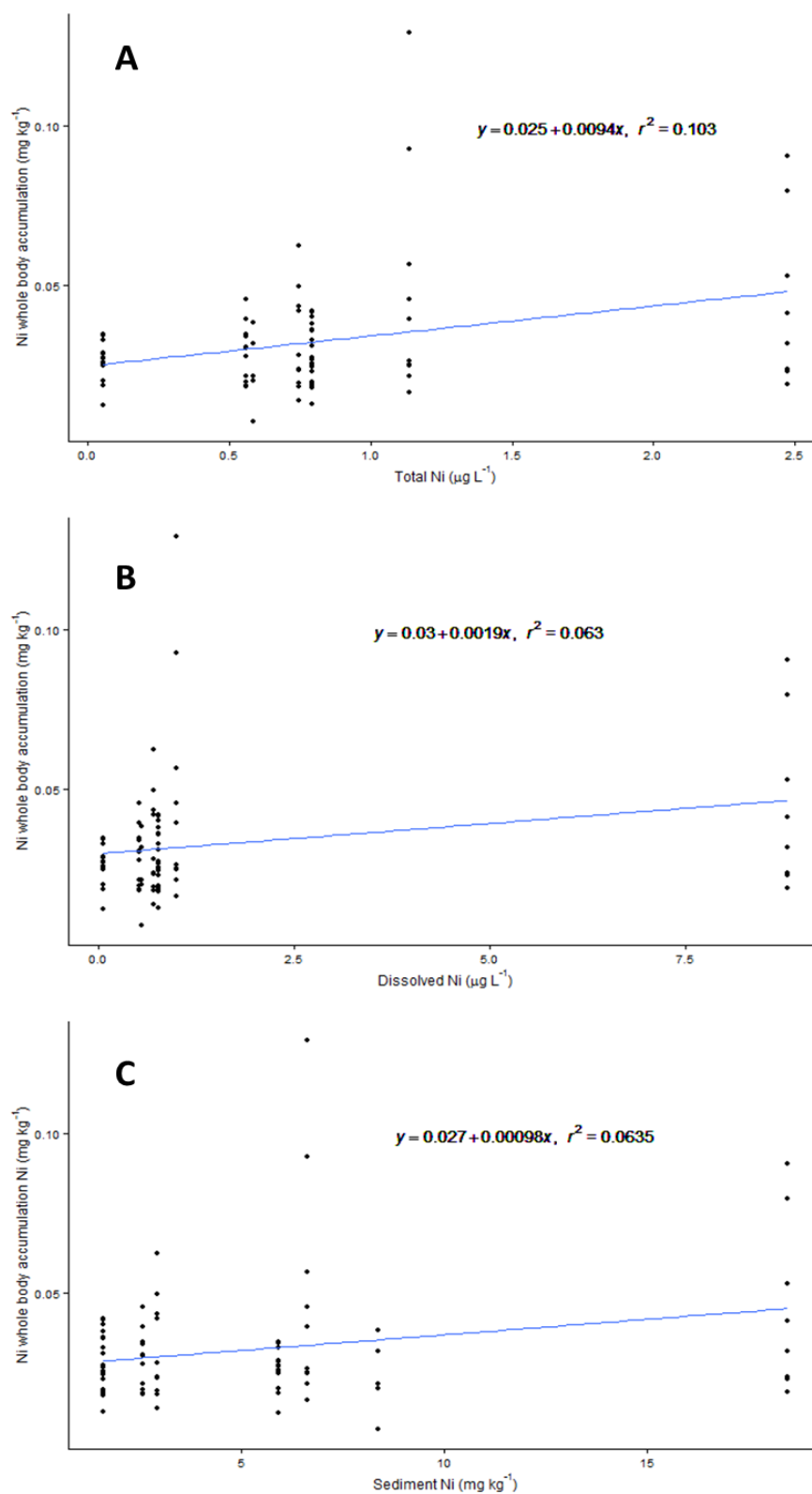


Figure 5.3 Linear correlations between fish Al accumulation (mg kg⁻¹) and A) total water Ni (μg L⁻¹), B) dissolved water Ni (μg L⁻¹), and C) sediment Ni (mg kg⁻¹) for data collected at both 96 h and 9 d. (Pearson's product-moment correlation, total p < 0.01, dissolved p = 0.03, sediment p = 0.03).

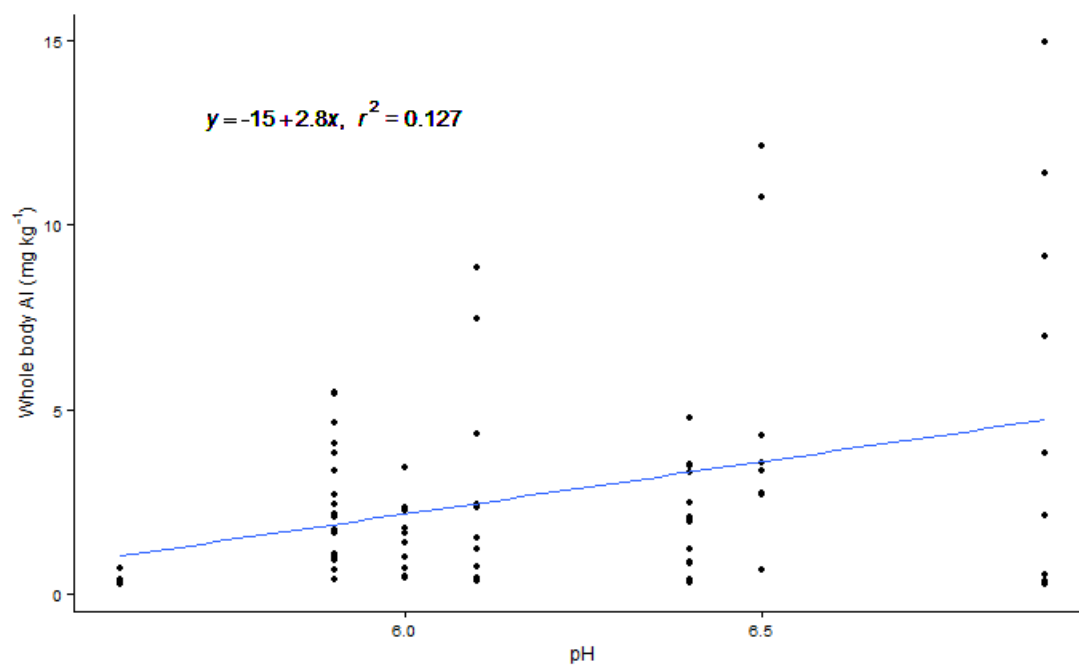


Figure 5.4 Linear correlation between fish Al accumulation (mg kg⁻¹) after both exposure periods and levels of pH in caging streams (Pearson's product-moment correlation, $p < 0.01$).

5.3.4 Na⁺/K⁺ ATPase

There were no significant differences in NKA activity between caged streams or exposure time (96 h and 10 d) and the fish collected from the source (two-way ANOVA). The closest stream to show a significant difference from the source was JC ($p = 0.66$).

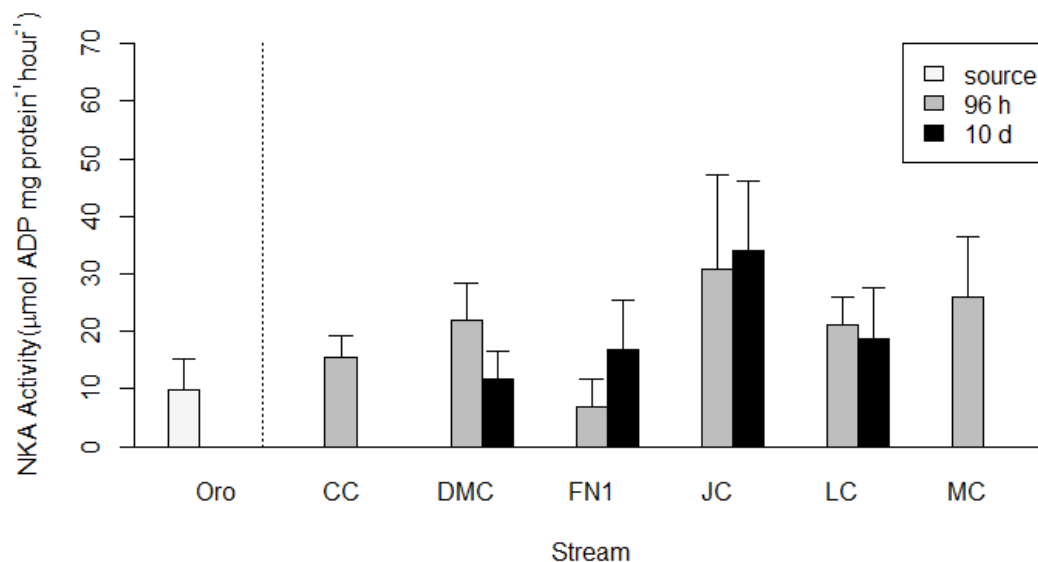


Figure 5.5 Measures of NKA activity ($\mu\text{mol ADP mg protein}^{-1} \text{hour}^{-1}$) in the gills at 96 hour and 10 day collection periods for source (Oro) and caging study streams (n-values in Table 5.5).

Table 5.5 N-values for NKA and LPO biochemical biomarker assays.

Stream	96 hour	10 day
CC	7	0
DMC	4	2
FN1	3	4
JC	4	4
LC	6	3
MC	5	0
Oro	2	

5.3.5 Lipid peroxidation

Measures of TBARS were significantly different between streams; however, there were no overall significant differences as a result of time (two-way ANOVA, p -value < 0.01). The only stream significantly different from the source (Oro) was LC after 10 d ($p < 0.01$) (Fig 5.6). TBARS in LC after 10 d was also significantly higher than all other time points in all other streams (two-way ANOVA, p -value ≤ 0.01).

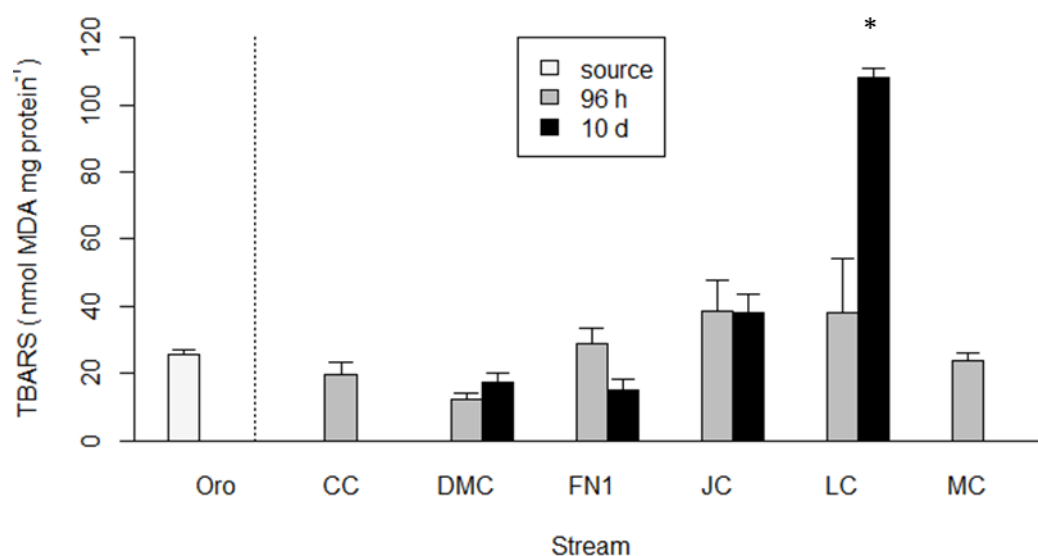


Figure 5.6 Measures of lipid peroxidation in the liver, as measured by MDA activity (nmol MDA mg protein⁻¹), at 96 hour and 10 day collection periods for source (Oro) and caging study streams (n-values in Table 5.5). Asterisk (*) denotes a significant difference relative to the source stream.

TBARS in fish liver was also positively correlated with stream pH (Fig. 5.7; Pearson's product-moment correlation, $p < 0.01$, $r^2 = 0.11$), but was not correlated to means of total water, dissolved water, or sediment trace element concentrations; nor was it correlated to NOM or total oxygen reactive (redox) metals (Cr, Cu, and Fe) in total water or sediments. However, the correlation between TBARS and mean dissolved redox metals fell just below significance ($p = 0.05$) (Table 5.6).

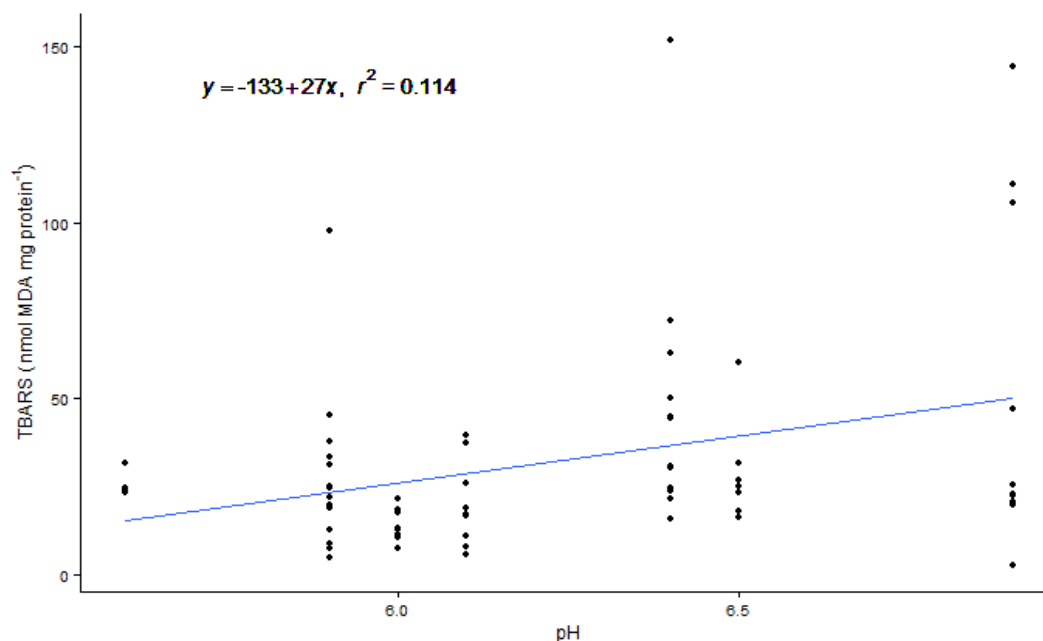


Figure 5.7 Linear correlation between measures of lipid peroxidation in the liver (TBARS) measured by MDA activity (nmol MDA mg protein⁻¹) and levels of pH in caging streams (Pearson's product-moment correlation, $p < 0.01$).

Table 5.6 Results of Pearson's product-moment correlations in relation to TBARS (nmol MDA mg protein⁻¹).

Variable	p-value	r ² value
Oxygen reactive species sediment	0.28	0.018
Oxygen reactive species total	0.20	0.026
Oxygen reactive species dissolved	0.05	0.061
pH	< 0.01	0.114
NOM	0.11	0.040
Mean sediment trace element concentrations	0.23	0.022
Mean total water trace element concentrations	0.34	0.014
Mean dissolved water trace element concentrations	0.07	0.051

The descriptor 'oxygen reactive species' in this instance refers to metals (Cr, Cu, Fe) with the capacity to directly generate reactive oxygen species, as opposed to other metals which may induce reactive oxygen stress but through less direct mechanisms.

5.4 Discussion

The results of this chapter have shown that trace element burdens in inanga did not change despite exposure to a range of streams that differed in their trace element composition. The lack of correlations between body burden and water/sediment concentrations for most trace elements measured (with Al and Ni being the exceptions) also indicates there may be other variables affecting accumulation. Further support for this is seen with biomarker analysis; where aside from TBARS at LC after 10 d (Fig. 5.5), there were no significant differences in measured biomarkers (TBARS and NKA). The pH of streams may have also played a significant role in toxicity as both whole body Al accumulation and measures of TBARS correlated significantly with pH. As Al was the only trace element to have significantly accumulated in this study, it is the focus of most of this discussion. However, it is also likely that other trace elements impacted final body burdens. For example, Fe has a strong influence on the ability of Al to bind to NOM (Nierop et al., 2002). As such, the amount of Fe present will influence the amount of bioavailable Al. This is only one example among a number of ways in which other trace elements can interact to influence availability (Norwood et al., 2003; Pandey et al., 2008). Determining the effect of these mixtures on trace element accumulation in inanga should be a future focus of research in examining the field responses of inanga to contaminated waters. Furthermore, as highlighted in Chapter 4, factors such as dietary metal exposure will also have a significant impact on trace element burden in inanga.

5.4.1 Whole body trace element accumulation

Total Al and Ni water concentrations were positively correlated to body burdens in this study. This contrasts to the findings in Chapter 4 where no such correlations between water levels and trace element body burdens could be identified. This is likely due to the fact that there were more streams in this study, leading to a greater statistical power. Although there were no significant differences in Ni body burden as a function of exposure stream in this study, it is possible that Ni accumulation could be predicted based on any of the three environmental measures (total water, dissolved water, and sediment trace

element concentrations). This suggests that making predictions regarding Ni toxicity would align with BLM approach (Niyogi and Wood, 2004). This is consistent with previous research on the development of Ni BLM's, which have been shown to be reliable and predictive (Schlekat et al., 2010; Schroeder et al., 2010).

Al was also the only metal to show significantly increased body burdens across streams, with LC and MC (Fig. 5.2) having the greatest body burdens. Changes in Al body burdens were significantly positively correlated with total water Al concentrations. This contrasts with Al toxicity in algae which was found to directly relate to dissolved Al (Parent and Campbell, 1994). However, a subsequent study with similar algae found that Al toxicity was no longer proportional to the free ion concentration as a consequence of NOM (Parent et al., 1996). This suggests dissolved Al concentrations did not correlate to accumulation in this chapter due to complexation with NOM. Al has previously been shown to complex with NOM (Linnik and Zhezherya, 2013), and Al body burdens were significantly correlated with NOM levels in this study. Filtration through a 0.45 μm filter is the functional definition of dissolved (i.e. free and ionic metal), but this is not always true. Some colloidal NOM is sufficiently small to pass through a 0.45 μm filter and as such some of the Al considered to be dissolved will actually be bound to NOM, thus giving a false perspective of Al bioavailability (Andren and Rydin, 2009). For example, 15-70% of dissolved Ag was associated with colloidal NOM in a study of natural waters in Texas (Wen et al., 1997). This makes predicting Al accumulation based on dissolved Al more complex. The free-ion activity model (FIAM) proposed by Morel (1983), and which the BLM approach is based on, states that the accumulation of trace elements directly relates to the amount present as reactive species (i.e. free ions). This FIAM, however, does not work under all situations. For example, Al toxicity in algae was not proportional to free Al ion (Al^{3+}) (Parent et al., 1996), a finding that is consistent with the lack of relationship between dissolved Al and Al accumulation in the current study. The correlation with total Al, rather than dissolved Ag, suggests that accumulation may partly represent Al that is adsorbed rather than absorbed. This could represent Al bound to surface mucus (Winter et al., 2005), or sorption of Al-NOM complexes to epithelial surfaces (Parent et al., 1996). Alternatively, other forms of Al, apart from that which is 'dissolved', may be bioavailable to inanga. This second possibility is less likely, with no evidence of, for example, Al-NOM complexes, being bioavailable to fish or other aquatic animals (Gensemer and Playle, 1999).

Al has been shown to accumulate on gill surfaces (Monette and McCormick, 2008), which was also supported by differences in whole body *versus* incomplete fish samples in Chapter 4. Additionally, whole body Al accumulation has been shown to be lower than gill Al concentrations, due to a dilution effect (Wilson, 2012). This suggests that measurements of tissue specific Al burdens may have been a more informative measure than whole body accumulation in this study. Determining trace element accumulation in the gills and liver would have been a useful endpoint to measure; however, inanga are very small and there was insufficient tissue to run both metal burden and biochemical assays.

5.4.2 The influence of pH

The bioavailability of Al has been shown to change abruptly with small changes in pH, making predictions of Al bioaccumulation difficult (Neville and Campbell, 1988). This may be further complicated by changes in pH at the boundary layer of the gill due to the excretion of ammonia, carbon dioxide, and protons (Playle, 1998). Specifically, carbon dioxide excretion can decrease the pH in this boundary layer, depending on the buffering capacity of the surrounding water, through the production and excretion of protons after conversion to HCO_3^- by carbonic anhydrase (CA) (Playle, 1998). This decrease in pH can lead to an increase in Al bioavailability (Gensemer and Playle, 1999). However, Al bioavailability changes in a parabolic manner with pH, with highly basic waters also having increased Al bioavailability (Gensemer and Playle, 1999). As pH in both LC and MC were higher than that in Oro, it is possible that the significant accumulation of Al in these streams was due to these pH differences. Consequently, shifts in bioavailability due to pH are important considerations when attempting to predict toxicity.

Although Al has been shown to act as a dual respiratory and ionoregulatory toxicant (Neville and Campbell, 1988; Playle et al., 1989), it is a more effective respiratory toxicant at higher pH (i.e. a pH of 5.2 was more toxic than pH of 4.8 and 4.4 in Playle et al., 1989). All of the streams in this study were less acidic than those in the Playle et al. (1989) study. The lack of a respiratory toxicity endpoint in this study (e.g. oxygen consumption as per Chapter 6) may have therefore precluded identification of significant toxic effects of the caging study on inanga.

The only toxic impact identified in the current study was a significant increase in liver TBARS activity in LC after 10 days (Fig. 5.5). This increase may have been a result of differences in pH, as LC was less acidic than the source (Oro) by more than 1 pH unit (Table 5.1). This is supported by a significantly positive correlation between pH and TBARS (Fig. 5.6). As Al was the only metal to significantly accumulate across streams, it is likely this increase in TBARS at LC was a result of pH related changes in the bioavailability of Al. Levels of total water Al (approximately $100 \mu\text{g L}^{-1}$) similar to those in LC have also been shown to induce oxidative stress in roach at a pH of 8 (Jolly et al., 2014). This supports the idea that pH is mediating changes in Al bioavailability and thus toxicity. It is also possible that the acid-base challenges resulting from moving fish from the most acidic stream (Oro) to the least acidic (LC) may have caused this increase in oxidative stress. More research is necessary to identify whether pH alone or in combination with Al is responsible for this increase in TBARS. In addition to metals, pesticides, organic hydro-peroxides, and organochlorines have also been shown to result in increased TBARS (Firat et al., 2011; Kappus, 1987; Lemaire et al., 2010). It is possible that other contaminants were present in LC and went unmeasured; however, LC was not any closer to agricultural or urban point sources than other caged streams, suggesting this may not be the case.

5.4.3 NKA

There were no significant differences in NKA activity as a result of exposure stream or time (Fig. 5.4). NKA inhibition is metal-dependent, with metals like Cu and Ag shown to greatly reduce NKA activity (Li et al., 1998; Morgan et al., 1997), and Fe having less of an effect (Lappivaara and Marttinen, 2005). Impacts on NKA activity are also known to be concentration dependent, so levels of trace elements in this caging study may not have been high enough to affect NKA activity. For example, acute Al exposures (2 to 6 days) of approximately $190 \mu\text{g L}^{-1}$ were shown to decrease gill NKA activity in Atlantic salmon (*Salmo salar*) in an acidic (pH 5.6-5.8) field setting, while laboratory exposures of $72 \mu\text{g L}^{-1}$ Al (pH 6.3-6.6) did not affect NKA activity (Monette and McCormick, 2008). The differences in NOM (1.5 mg C L^{-1} in LC versus 4.2 mg C L^{-1} in Oro) may have been responsible for mitigating the effects of Al toxicity, as described in Section 5.4.2. It is also possible that the trace elements

typically responsible for impacting NKA in freshwater fish (Ag, Cu) were not at sufficiently high levels to cause effects on NKA activity. Additionally, Oro had relatively high levels of trace elements, so inanga may have already been acclimated. As different isoforms of NKA appear to have different metal sensitivities *in vitro* (M.A. Urbina, J.V. Hill and C.N. Glover, unpublished data), determining the isoforms present in each stream may have helped elucidate whether acclimation had occurred.

5.4.4 Regulatory context

As inanga are not sediment-associated fish, sediments are unlikely to have had a large effect on metal accumulation. This is supported by fish accumulating more Al in streams of lower sediment Al concentrations. It is possible that sediments burdens could influence water trace element concentrations; however, only levels of Ni in sediments were correlated to total water concentrations. Instead, it is possible that sediments influenced trace element concentrations of prey. Inanga are opportunistic feeders (Becker and Laurenson, 2007) exposing them to a range of prey, some of which may inhabit or ingest sediment. However, there is little evidence for bioaccumulation of the key elements in this study (Al and Fe) (Winterbourn et al., 2000). While accumulation due to sediment exposure cannot be ruled out, it is unlikely they had a large influence on resulting body burdens in inanga.

As outlined above, it is likely that changes in pH played a role in the bioavailability of Al, impacting both accumulation and biomarker responses. Current ANZECC water guidelines for Al are based on varying pH, with a trigger value of $0.8 \mu\text{g L}^{-1}$ Al calculated to protect 95% of aquatic biota at $\text{pH} < 6.5$. However, at a pH greater than this, the trigger value jumps to $55 \mu\text{g L}^{-1}$ Al (Table 5.2; ANZECC/ARMCANZ, 2000). All streams in this exposure had values of Al higher than these trigger values, which should then 'trigger' a management response. As fish accumulated Al in streams with lower Al concentrations, it is clear that pH, physiology, or other variables are involved. A few streams also had Fe (all streams excluding JC and MC) and Zn (FN1 and Oro) water concentrations above their trigger values, with no accumulation. Of the other trace elements measured (As, Cr, Cu, and Ni), most streams were below trigger values. These results suggest these trigger values

would likely be sufficient at preventing accumulation. However, the proportion of streams exceeding these values suggests the management response they should 'trigger' is not sufficient.

Chapter 6: Evaluating Fe and Zn toxicity in inanga (*Galaxias maculatus*) through *in vivo* laboratory exposures

6.1 Introduction

Laboratory toxicity experiments allow for better control of variables than *in situ* field studies. Through isolating specific variables, interactions are removed and mechanisms of toxicity can be elucidated. For waterborne trace element exposures, it is now well-established that the gill is the most toxicologically significant tissue (Paquin et al., 2002). This understanding has led to predictive models using water chemistry to predict metal bioavailability and therefore toxicity (i.e. BLM; Di Toro et al., 2001). As accumulation has been shown to relatively accurately predict toxicological consequences to aquatic biota (Clearwater et al., 2002; Di Toro et al., 2001; Norwood et al., 2003), determining uptake is necessary for predicting toxic effects. However, determining the specific mechanisms of uptake, physiological responses, and species-specific variations in accumulation is still required for many metals. Sensitivity to metal toxicity has also been shown to vary depending on species. For example, mottled sculpins (*Cottus* spp.) were shown to be more sensitive to Cd, Cu, and Zn than rainbow trout (*Onchorhynchus mykiss*) (Besser et al., 2007). Species-specific physiology has already been suggested as a major determinant in Cu toxicity, with variability across species having nearly four magnitudes difference depending on species and life-cycle stage (Grosell et al., 2007). Consequently it is important that relationships between metal exposures and toxicity in fish are adequately determined to ensure that regulatory tools based on such relationships are adequately protective.

Little is known regarding metal toxicity in inanga. As such, the applicability of predictive models, like the BLM, to this species is uncertain. Inanga have been shown to have relatively plastic physiological responses to changes in their environment (Glover et al., 2012; Urbina et al., 2011). Inanga also have some unusual physiological characteristics (e.g. significant use of skin as a gas exchange surface (Urbina et al., 2011) and a tolerance to acid water (Glover et al., 2012)) which may make them either more or less susceptible to metal toxicity. The use of skin as a respiratory surface, for example, may make them more susceptible to Fe toxicity, as Fe is a respiratory toxicant (Dalzell and MacFarlane, 1999). Tolerance to acidic waters could also place them in streams in which metal bioavailability is higher than more pH-neutral streams (Winter, 2005). However, the ability to withstand acid waters also suggests these fish have strong osmoregulatory abilities, which may help inanga avoid some of the negative ionoregulatory consequences of metal toxicity.

The current study examines the effect of Fe and Zn on metal uptake and toxicity in inanga. These metals were chosen as they are of environmental relevance in inanga natural habitats. For example, Fe is highly elevated in acid mine drainage-impacted streams of New Zealand's West Coast and in Chapter 4 whole body Fe burdens significantly increased in transplanted fish. Zn is also specifically important for inanga as it is typically elevated in urban waters (O'Sullivan et al., 2012), many of which are potential inanga habitats. Additionally, although both are essential elements for fish (Wood, 2012), they work via different modes of toxicity (Dalzell and MacFarlane, 1999; Hogstrand and Wood, 1996). Toxicity due to increased Fe exposure most often results from gill clogging and damage, leading to a reduced available surface area and increased diffusion distance for ion exchange and respiration (Dalzell and MacFarlane, 1999; Peuranen et al., 1994). As such, waterborne Fe exposure is considered more toxic than dietary exposure (Bury and Grosell, 2003a; Bury et al., 2001; Dalzell and MacFarlane, 1999). Zn is thought to cause toxicity through disturbances to ion balance (Hogstrand et al., 1995). Specifically, Zn has been shown to compete with Ca^{2+} uptake at the gills, subsequently disturbing gill ion regulation (Hogstrand et al., 1994; Hogstrand et al., 1995).

Physiological and biochemical biomarkers as a means of revealing the toxic effects of metals have been broadly used in freshwater fish (van der Oost et al., 2003). Among the most relevant of endpoints for Fe and Zn is oxygen consumption. Both Fe and Zn exposures have resulted in increased oxygen consumption rates in fish (Lappivaara et al., 1995; Peuranen, 1994). Oxygen consumption and ammonia production, as indices of metabolism and protein catabolism (Cech, 1990; Wright and Wood, 2012) respectively, were used in this study to determine metabolic costs. Additionally, both Fe and Zn have been shown to result in oxidative stress in freshwater fish (Hamoutene et al., 2000; Loro et al., 2012). As such, TBARS formation, as a biomarker of LPO, was measured after exposures. Ca^{2+} and Na^{+} influx were also measured in order to determine the impacts of Fe and Zn on specific ion transport (Wood, 1992).

This study aims to determine concentration-dependent Fe and Zn accumulation in a controlled laboratory study. Exposure concentrations were chosen based on low levels, below trigger values (1 mg L^{-1} Fe and $5 \text{ } \mu\text{g L}^{-1}$ Zn) (Table 5.2; ANZECC/ARMCANZ, 2000), environmentally relevant levels (5 mg L^{-1} Fe and $50 \text{ } \mu\text{g L}^{-1}$ Zn) measured in New Zealand streams (Ch. 4), and values close to the LC_{50} for other species (20 mg L^{-1} Fe (Dalzell and

MacFarlane, 1999) and 200 $\mu\text{g L}^{-1}$ Zn (Bradley and Sprague, 1985)). Additionally, biochemical and physiological biomarkers were measured in order to provide an understanding of the specific mechanisms of Fe and Zn toxicity in inanga. The working hypothesis being tested was that metal accumulation and other physiochemical parameters would relate to dissolved water concentrations.

6.2 Methods

6.2.1 Animal care

Adult inanga (*Galaxias maculatus*; size range 0.64 – 3.28 g) were collected and maintained as described in Section 2.1-2.2. All animal manipulations were approved by the Animal Ethics Committee of the University of Canterbury.

6.2.2 Experimental setup

Ninety-six hour Fe and Zn exposures, as described in Section 2.9.1, were performed at exposure levels of 1, 10, and 20 mg L^{-1} Fe and 5, 50, and 200 $\mu\text{g L}^{-1}$ Zn. Stock solutions of 2.5 and 15 g L^{-1} Fe (as FeCl_3) and 0.11 and 1.1 g L^{-1} Zn (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) were prepared in Milli-Q water with 70% trace grade nitric acid added at a ratio of 50 μL per 15 mL. For Fe, a volume of 1.2 mL of the 2.5 g L^{-1} Fe stock, 2 mL of the 15 g L^{-1} Fe stock, and 4 mL of the 2.5 g L^{-1} Fe stock were each added to 3 L of aquarium water for concentrations of approximately 1, 10, and 20 mg L^{-1} Fe concentrations, respectively. For zinc, a volume of 200 μL of the 0.11 g L^{-1} Zn stock, 150 μL of the 1.1 g L^{-1} Zn stock, and 550 μL of the 1.1 g L^{-1} Zn stock were each added to 3 L of aquarium water for concentrations of approximately 5, 50, and 200 mg L^{-1} Zn concentrations, respectively. The pH of exposure solutions were checked after the addition of metals, and no pH differences were determined. A control of aquarium water was included in each exposure series. Waters were given 24 hours to equilibrate before fish were added. Water was under constant aeration, and a 50% water change (with equilibrated water) was conducted after 48 hours. Fish were not fed during the exposure period.

Fish (mass ranging 0.64-3.28 g) were left in exposure containers for 96 hours and water samples were collected as described in Section 2.9.1. Fish were removed for determination of physiological and biochemical endpoints as per Sections 2.8.1, 2.8.2, 2.9.2, and 2.9.3. Fe and Zn concentrations of water samples was then determined *via* ICP-MS as per Section 2.7.2.

6.2.3 Physiological endpoints

After 96 hours, fish were placed in closed-box respirometers for one hour in order to measure metabolic rates as described in Section 2.8.2. Water samples were also taken from respiration chambers before initial and after final respiration samples, in order to measure ammonia production via the salicylate method (API Ammonia Test Kit, Section 2.8.3).

6.2.4 Dissections & digestions

Fish were then removed from respirometers, euthanized, weighed, and dissected as per Section 2.2. Both gill and liver were removed for biochemical analyses while the rest of the body was digested per Section 2.3 before being run on ICP-MS for the determination of Fe and Zn concentrations.

6.2.5 Biochemical assays

Subsamples of liver tissue were assayed for TBARS production using the method described in Section 2.7.1. Subsamples were also measured for total protein via the Bradford assay as described in Section 2.7.3. The rate of MDA formation was calculated as nmol MDA formed per milligram of protein (nmol MDA mg protein⁻¹). Gills were removed for NKA analysis; however, there were problems with the assay, so results were not included in this chapter.

6.2.6 Ca^{2+} influx

Following 96 hour exposures to 20 mg L^{-1} Fe and $200 \text{ } \mu\text{g L}^{-1}$ Zn, with water identical to composition of Section 2.8.1, Ca^{2+} influx was determined in exposed fish with protocols similar to Hogstrand et al. (1995). Fish were placed in flux chambers (4-L ziplock bags, double-bagged in case of leakage) filled with corresponding exposure waters. Fish were acclimated for two hours (Harley and Glover, 2013) before $200 \text{ } \mu\text{L}$ (containing $20 \text{ } \mu\text{Ci}$) of $^{45}\text{Ca}^{2+}$ radioisotope was added to each bag. Fish manipulations and water samples were conducted as described in Section 2.4.1. Fish were weighed and digested in 10 mL of 2N Analar grade nitric acid in a 60°C for approximately 72 hours before being analyzed for $^{45}\text{Ca}^{2+}$. Samples were prepared for analysis with 2 mL of digest added to 15 mL of fluor (Perkin Elmer Ultima Gold). Water samples had 5 mL of fluor added, and both samples were left for approximately 4 hours in the dark before being run on a liquid scintillation analyzer (Perkin Elmer Tricarb 2910 TR) for total $^{45}\text{Ca}^{2+}$. Resulting cpm from water samples were used to calculate specific activity *via* Equations 2.1 & 2.2 (Section 2.4.1).

For fish digests, a calcium quench curve was constructed in order to correct for the effect of fish tissue attenuating the isotope signal. This was done *via* the external standards ratio approach (similar to Rogers and Wood, 2004).

6.2.7 Na^+ influx

After Fe and Zn exposures (Section 2.8.1), fish were placed in bags similar to Section 6.2.6. To each bag, $160 \text{ } \mu\text{L}$ (containing $20 \text{ } \mu\text{Ci}$) of $^{22}\text{Na}^+$ radioisotope was added. Methods were similar to Section 2.5.1, except that a high Na^+ rinse was used instead of a high Ca^{2+} rinse. After being rinsed and euthanized, whole fish and water samples were directly measured for $^{22}\text{Na}^+$ via gamma counter (Perkin Elmer Wallac Wizard 1470). Resulting cpm from water samples were used to calculate specific activity *via* Equations 2.1 & 2.2 (Section 2.4.1).

Total Na^+ levels in the exposure solutions were determined via flame spectrophotometry (Sherwood Instruments). A standard curve, fitting at least $r^2 = 0.98$ was constructed using known amounts of Na^+ dissolved in distilled water. This curve was then used to calculate Na^+ levels in each sample.

6.2.8 Statistical analyses

Data were analyzed as described in Section 2.11, with each endpoint being analyzed using a one-way ANOVA. All statistical analyses were conducted using the R software (Version 2.15.0) with $p < 0.05$ considered significant.

6.3 Results

6.3.1 Water chemistry

Measured metal concentrations of Fe were close to nominal concentrations; however, actual Zn concentrations were higher than nominal values (Table 6.1). Additionally, dissolved concentrations of Fe were significantly lower than total, while dissolved Zn concentrations were similar to total (Table 6.1).

Table 6.1 Water metal concentrations for exposures (mean \pm standard error of the mean, $n = 4$ each)

Metal (unit)	nominal	total	dissolved
Fe ($\mu\text{g L}^{-1}$)	0	1.3 ± 0.1	1.2 ± 0.5
	1000	700 ± 10	2.7 ± 0.4
	10000	8100 ± 160	10.8 ± 0.7
	20000	16400 ± 970	44.0 ± 20.0
Zn ($\mu\text{g L}^{-1}$)	0	6.1 ± 0.9	5.6 ± 0.6
	5	25.2 ± 1.2	23.3 ± 1.3
	50	66.5 ± 1.3	60.7 ± 1.8
	200	222.5 ± 1.0	218.7 ± 1.2

6.3.2 Fish metal accumulation

Neither Fe nor Zn body burdens were significantly different across exposure groups (one-way ANOVA). In fact, the 200 $\mu\text{g L}^{-1}$ Zn treatment resulted in the lowest accumulation across all Zn exposure groups, including the control (Fig. 6.1, mean = $32.0 \pm 1.8 \text{ mg kg}^{-1}$).

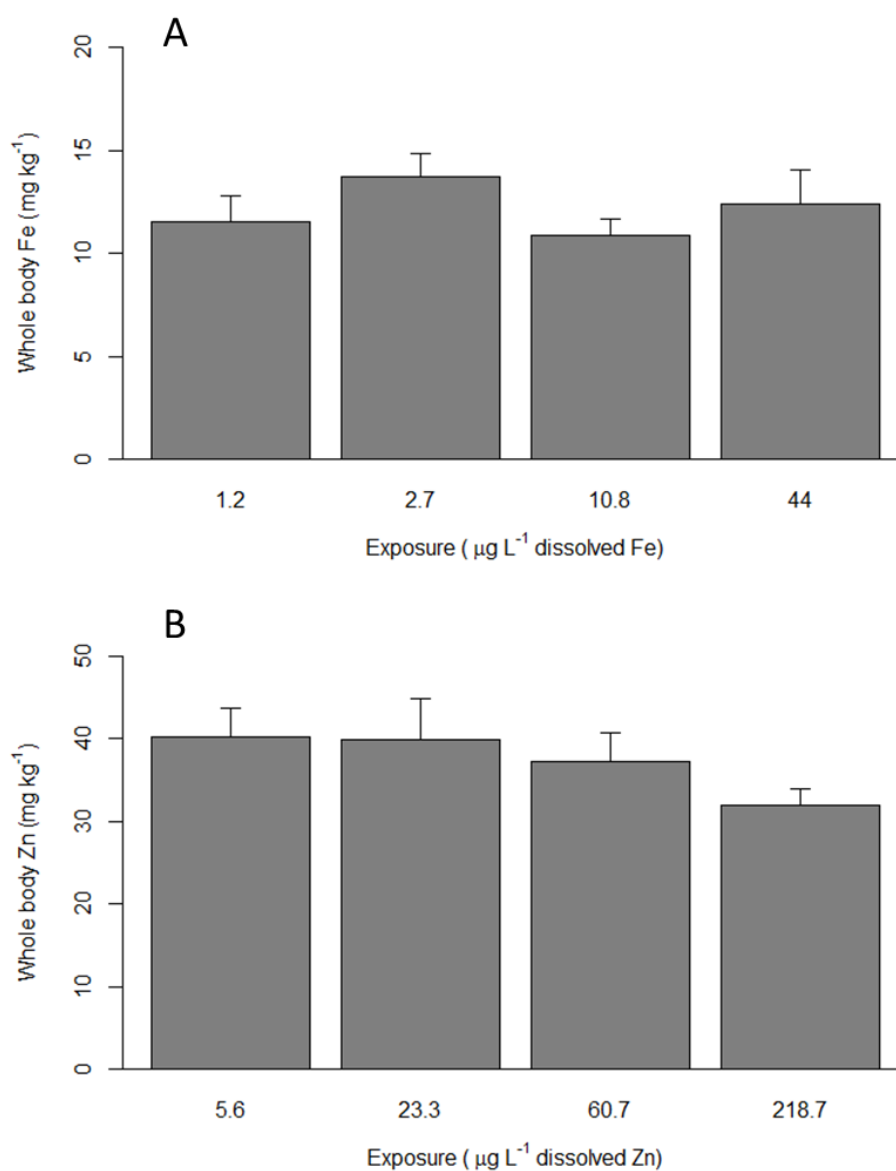


Figure 6.1 Whole body accumulation (mg kg^{-1}) for Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L^{-1} Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

6.3.3 Respiration

Metabolic rates (MO_2 , $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$) for 20 mg L^{-1} Fe (dissolved Fe: $44 \mu\text{g L}^{-1}$) exposures were significantly higher than those for control fish and 10 mg L^{-1} Fe (dissolved Fe: $10.8 \mu\text{g L}^{-1}$) exposures (Fig. 6.2, one-way ANOVA, $p = 0.01$). However, there was no significant difference between the 1 mg L^{-1} Fe (dissolved Fe: $2.7 \mu\text{g L}^{-1}$) exposures and any of the other treatments (one-way ANOVA, $p > 0.05$). There were no significant differences in metabolic rates (MO_2 , $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$) between treatment groups in Zn exposures (one-way ANOVA, $p > 0.05$).

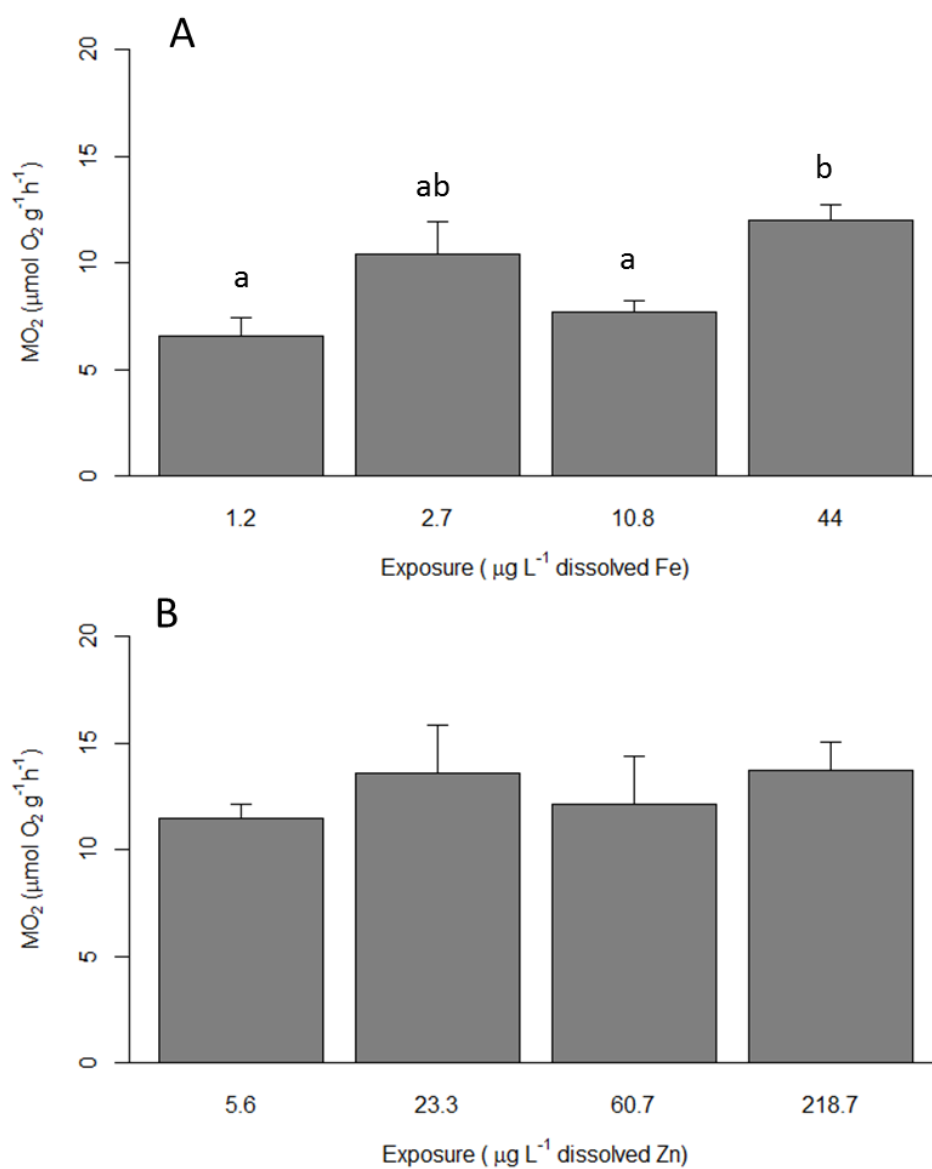


Figure 6.2 Metabolic rates (MO₂, µmol O₂ g⁻¹ h⁻¹) as determined *via* closed box respiration after Fe (A) and Zn (B) exposures (n = 3 for each group, except 2.7 mg L⁻¹ Fe which only had 2). Bars sharing letters are not statistically significantly different (one-way ANOVA).

6.3.4 Ammonia

There were no significant differences in ammonia production across both Fe and Zn exposure treatments (Fig. 6.3, one-way ANOVAs, $p > 0.05$). There was little change in ammonia production with Fe exposure; however, there was a non-significant decrease at the $10.8 \mu\text{g L}^{-1}$ concentration (Fig. 6.3a). Although ammonia production in Zn treatments initially increased with increasing Zn concentrations, these changes were non-significant, and ammonia production decreased again at the highest Zn exposure (Fig. 6.3b).

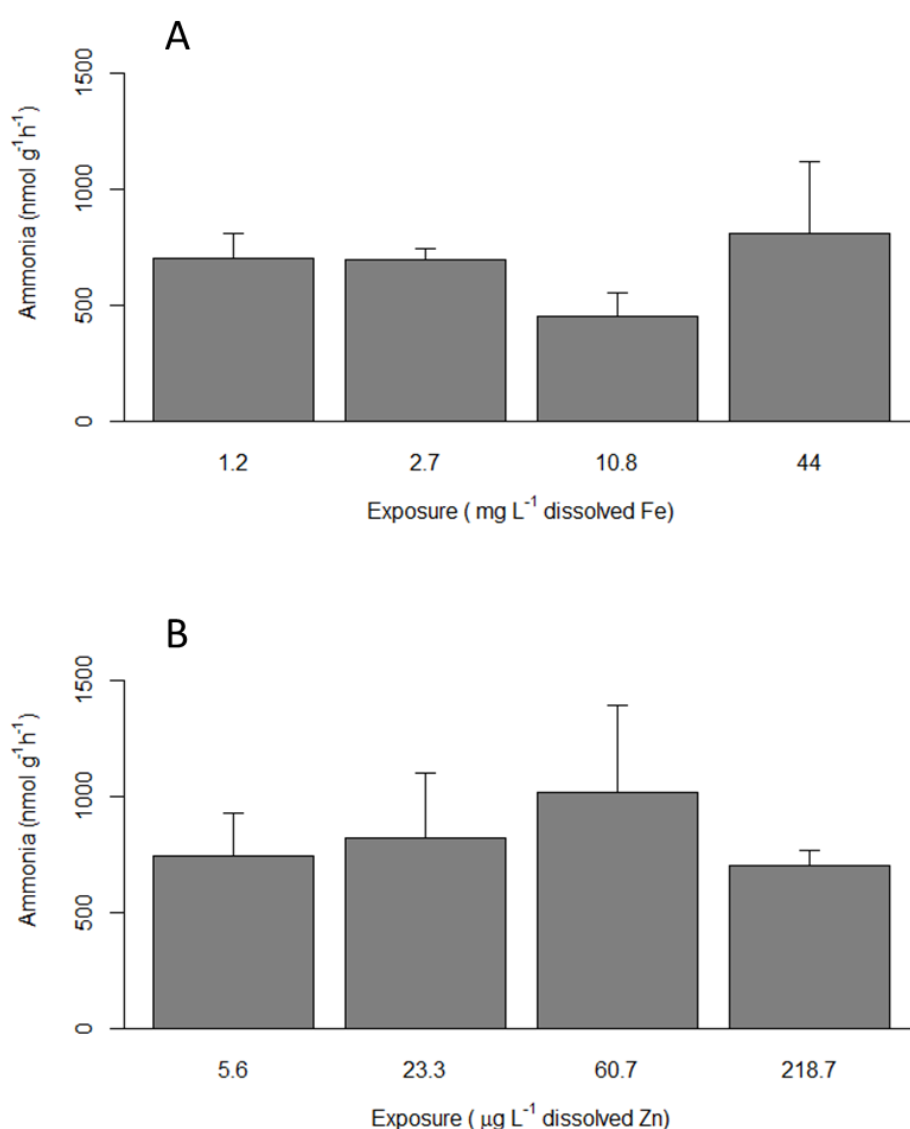


Figure 6.3 Ammonia production ($\text{nmol g}^{-1} \text{h}^{-1}$) measured after Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L^{-1} Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

6.3.5 Lipid peroxidation

There were no significant differences in lipid peroxidation as measured by the production of TBARS (nmol MDA mg protein⁻¹) across both Fe and Zn exposure treatments (Fig. 6.4, one-way ANOVA, $p > 0.05$). However, there was an apparent, non-significant, increase at 10.8 mg L⁻¹ Fe and 218.7 µg L⁻¹ Zn (Fig. 6.4).

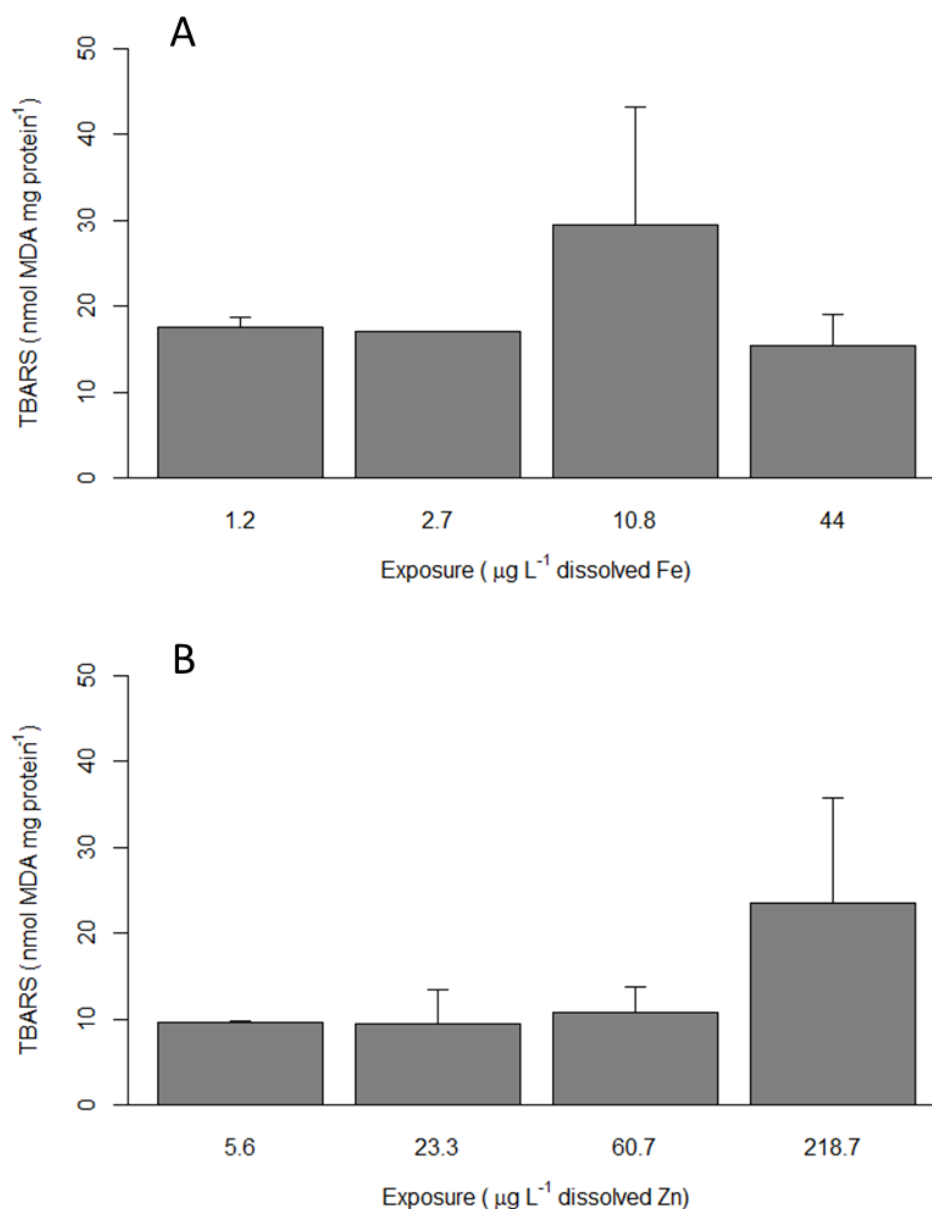


Figure 6.4 Levels of oxidative stress as measured by TBARS production (nmol MDA mg protein⁻¹) in the liver after Fe (A) and Zn (B) exposures ($n = 3$ for each group, except 2.7 mg L⁻¹ Fe which only had 2). There were no significant differences between treatment groups (one-way ANOVA).

6.5.6 Ion influx

Ca^{2+} influx ($\text{nmol g}^{-1} \text{h}^{-1}$) was not significantly different between the control and fish exposed to $44 \mu\text{g L}^{-1}$ Fe or $218.7 \mu\text{g L}^{-1}$ Zn (Fig 6.5, Student's t-test). Na^{+} influx ($\text{nmol g}^{-1} \text{h}^{-1}$) was also not significantly different between exposure treatments (Fig 6.6, Student's t-test). However, differences in Na^{+} influx fell just below significance ($p = 0.07$) between control and Fe exposed fish.

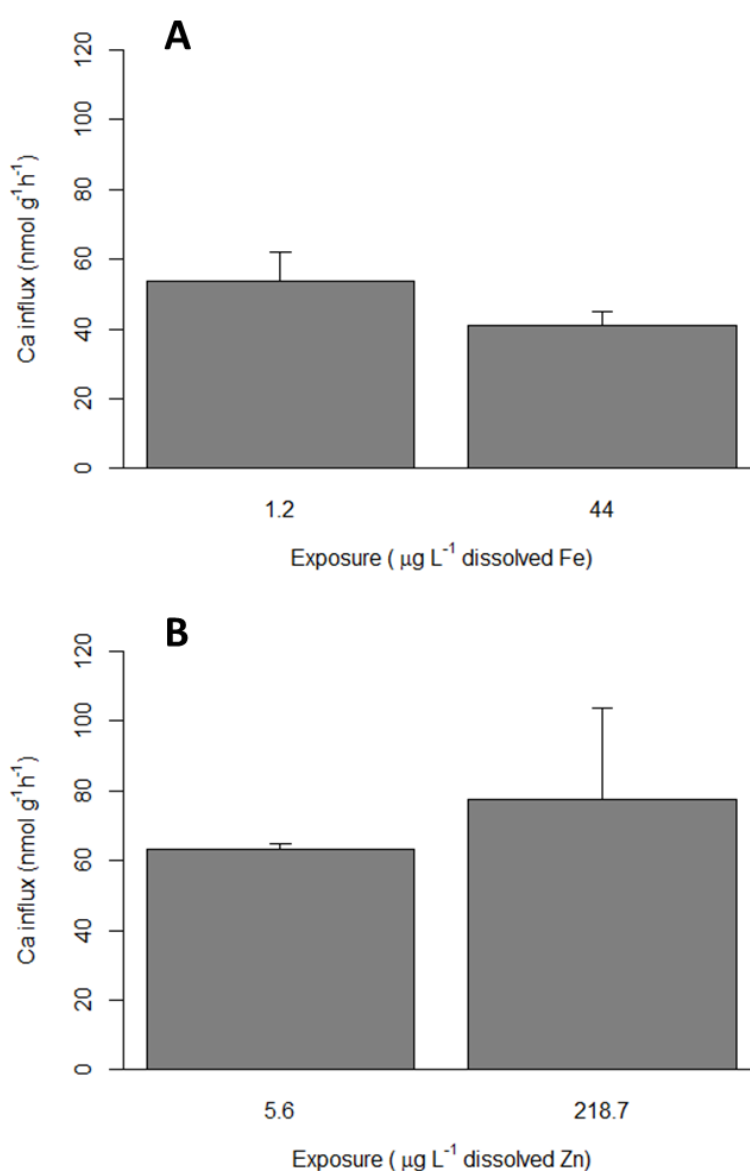


Figure 6.5 Ca^{2+} influx ($\text{nmol g}^{-1} \text{h}^{-1}$) in fish after 96 h exposures to A) Fe ($n = 7$) and B) Zn ($n = 8$). There were no significant differences between treatment groups (Student's t-test).

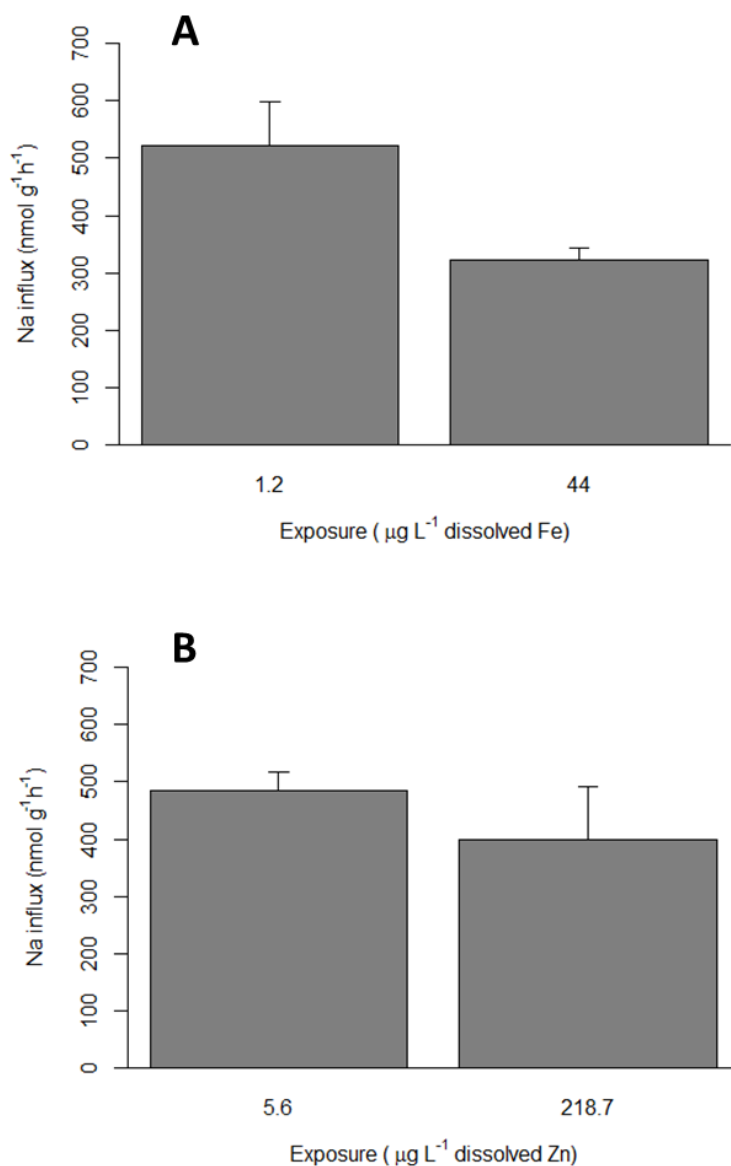


Figure 6.6 Na^+ influx ($\text{nmol g}^{-1}\text{h}^{-1}$) in fish after 96 h exposures to A) Fe ($n = 8$) and B) Zn ($n = 8$). There were no significant differences between treatment groups (Student's t-test).

6.4 Discussion

This study demonstrated the relative resilience of inanga in the face of Fe and Zn contaminated waters. The highest dissolved Fe level tested in this study ($44\ \mu\text{g L}^{-1}$) was similar to those previously shown to result in respiratory disruption in brown trout (*Salmo trutta*) (96 h exposure to $50\ \mu\text{g L}^{-1}$ soluble Fe) (Dalzell and MacFarlane, 1999). While there were significant alterations to metabolic rates after Fe exposures in this study (Fig. 6.2), they did not directly correspond to exposure levels. As Fe exposures did not show significant

accumulation, changes in TBARS, or modifications to ion transport, it suggests the only toxic effect of Fe in inanga at these exposure levels is respiratory impairment. In contrast, Zn exposures resulted in no effect on accumulation, respiration, or any other biological or biochemical biomarkers. The highest dissolved Zn exposure ($218.7 \mu\text{g L}^{-1}$) in this study was similar to the LC_{50} value ($170 \mu\text{g L}^{-1}$) for rainbow trout (*Salmo gairdneri*) after acute (96-120 h) exposures at a low hardness ($30 \text{ mg CaCO}_3 \text{ L}^{-1}$), pH of 7, and alkalinity of $8.4 \text{ mg as CaCO}_3 \text{ equivalents L}^{-1}$ (Bradley and Sprague, 1985). However, at similar pH and alkalinity, the LC_{50} jumped to $4,460 \mu\text{g L}^{-1}$ with increased hardness ($383 \text{ mg CaCO}_3 \text{ L}^{-1}$) (Bradley and Sprague, 1985). As hardness in the current study was $70 \text{ mg CaCO}_3 \text{ L}^{-1}$, the LC_{50} would be expected to be closer to the $170 \mu\text{g L}^{-1}$ value. As inanga were capable of surviving these levels of Zn for 96 h without accumulation or changes to toxic endpoints measured, it suggests they are relatively resilient to the toxic effects of Zn. Additionally, whole body Fe (mean 12.1 mg kg^{-1}) and Zn (mean 37.4 mg kg^{-1}) for all exposure criteria in this study were similar to control fish whole body Fe and Zn in previous studies: 11.2 mg kg^{-1} Fe (Carriquiriborde et al., 2004) and 37.7 mg kg^{-1} Zn (Spry et al., 1988). This suggests inanga are capable of preventing Fe and Zn accumulation in the face of hazardous exposure criteria.

6.4.1 Physiological and biochemical toxicity endpoints

The lack of whole body metal accumulation suggests inanga are capable of either preventing metal uptake or increasing excretion. However, had excretion increased, it would have come at a metabolic cost (McGeer et al., 2000b). While Fe exposures resulted in changes to metabolic rates, it is likely these changes were due to disruptions caused by Fe clogging at the gills (Dalzell and MacFarlane, 1999). The lack of consistent changes in oxygen uptake in response to increasing exposure levels may have been due to compensation by cutaneous oxygen uptake; which has already been shown to occur in this species (Urbina et al., 2012). Zn exposures did not result in metabolic changes, so it is unlikely that excretion was the means of preventing Zn accumulation in inanga. Examining gill histology and measuring gill Fe accumulation after Fe exposures would be an important step in determining the cause of respiratory impairments due to Fe exposures.

There were no significant differences in ammonia production as a result of either Fe or Zn exposures, suggesting there was no increase in protein metabolism due to exposures. Ammonia excretion is facilitated by the acidification of the apical gill boundary layer *via* apical NHE and CA activity (Wright and Wood, 2009). Guppies (*Poecilia vivipara*) exposed to Cu have previously shown an increase in ammonia production, likely due to interferences with NHE and subsequent changes in boundary layer pH (Zimmer et al., 2012). Acute Zn exposures in soft waters have also resulted in metabolic acidosis through increased ammonia production in the gills of rainbow trout (Spry and Wood, 1985). As there were no differences in ammonia production in this study, it suggests the pH of the boundary layer of the gills was not significantly disrupted and inanga did not suffer from metabolic acidosis. The inhibition of ammonia excretion is due to the inhibition of CA within the cell (Zimmer et al., 2012), as neither Fe nor Zn significantly accumulated in inanga, it is unlikely concentrations within the cell were high enough to inhibit CA.

Inanga did not appear to suffer from lipid peroxidation as a result of Fe and Zn exposures as there were no significant differences in TBARS within exposure groups (Fig. 6.5). TBARS values in African catfish after exposure to industrial runoff reached approximately 60 nmol mg protein⁻¹, while inanga TBARS values as a result of both Fe and Zn exposures were more similar to those found in the control catfish group (approximately 20 nmol mg protein⁻¹) (Farombi et al., 2007). This suggests that lipid peroxidation levels measured in inanga are normal, and that no significant additional lipid peroxidation was caused by Fe and Zn exposures. However, it is possible that these exposures impacted measures of oxidative stress that were not examined in this study. For example, the Atlantic killifish (*Fundulus heteroclitus*) showed an increase in ROS and alterations to antioxidant enzymes when exposed to elevated Zn concentrations (500 µg L⁻¹) for 96 h, suggesting an induction of oxidative stress (Loro et al., 2012). While there are numerous ways to measure oxidative stress in freshwater fish (GSH, CAT, protein carbonyls, etc.), each has its limitations, and none have been identified as a preferred methodology (van der Oost et al., 2003).

6.4.2 Whole body accumulation

Whole body Fe and Zn accumulation in the current study matched whole body accumulation of these metals in fish from the two caging studies (Ch. 4 & 5). Interestingly, fish from the first caging study (Ch. 4) accumulated significantly more Fe ($19\text{--}24\text{ mg kg}^{-1}$) in caged streams, which were lower in water Fe concentrations (ranging from $0.019\text{--}0.649\text{ mg L}^{-1}$), than the source stream. However, in this chapter they accumulated less Fe ($11\text{--}14\text{ mg kg}^{-1}$) in waters of much higher concentrations (up to 44 mg L^{-1} dissolved Fe). As discussed in Ch. 4, it is likely that diet played a role in this difference. Fish in laboratory exposures were not fed throughout the exposure, whereas fish in caging exposures had access to food, suggesting dietary uptake may have been the source of additional Fe. As mentioned in Section 5.4.3, pH was also likely to play an important factor in toxicological responses to exposure in the field. It is also possible that the Fe in laboratory exposures was less biologically available. The large differences between total and dissolved water Fe concentrations in laboratory exposure waters, and the considerably higher dissolved values in caged streams (Table 5.2), suggest this may be the case. It is possible that the use of FeCl_3 salt did not dissolve well enough, and that the use of another salt, such as FeSO_4 (similar to Dalzell and MacFarlane, 1999) may have been more appropriate.

6.4.3 Ion balance

The lack of significant differences in both Ca^{2+} and Na^+ fluxes suggests that neither Fe nor Zn significantly impact ion balance in inanga. This is an important finding as Zn is thought to compete with Ca^{2+} at the gill for uptake, leading to hypocalcemia (Spry and Wood, 1985). Should this mechanism differ in inanga, modelling based on this effect would then fail to accurately predict Zn toxicity in inanga. However, the lack of Zn accumulation may explain why there were no effects on Ca^{2+} transport. It is also possible that inanga are using their skin as an additional site of Ca^{2+} uptake. This has previously been shown to occur in rainbow trout (Marshall et al., 1992), and was recently shown in Chapter 7 for inanga. However, it is important to note that the results of Chapter 7 do show inhibition as a result of Zn exposure. It is possible that the gill is more capable of adjusting uptake rates to

account for Zn interferences than the skin, but this would require more research.

Determining the mechanisms by which Zn interacts with Ca^{2+} at both the skin and gill is an important step in elucidating the specific toxicological effects of Zn on inanga.

6.4.4 Future perspectives

Accumulation is seen as a good indicator of toxicity (Clearwater et al., 2002; Di Toro et al., 2001; Norwood et al., 2003); however, accumulation alone may not be enough to predict toxicity in inanga. The only toxic effect measured was a change in respiration due to Fe exposures, and that is likely explained by surface effects, not uptake. This study only measured a few toxicological endpoints. It is possible there were other physiological consequences to Fe and Zn exposures, as a result of avoiding accumulation, that were not measured. As mentioned above, examining the histology and measuring Fe content of the gills after Fe exposures would be an important step to determining if Fe was accumulating in gills, as has been shown in brown trout (Dalzell and MacFarlane, 1999). Additionally, altering the bioavailability of Fe and Zn in exposure waters, through the use of different Fe salts (FeSO_4) or altering water chemistry, in terms of hardness and acidity, may lead to increased accumulation. However, this study was intended to mimic environmental conditions, and as such, aquarium water with environmentally relevant Fe and Zn levels was used.

Overall, this study suggests inanga are capable of withstanding a range of Fe and Zn concentrations with only minor physiological, and no biochemical, impacts. The lack of Zn accumulation coincides with both *in situ* studies (Ch. 4 & 5), while the differences in Fe accumulation between chapters was likely due to differences in bioavailability or diet. It also appears inanga are capable of regulating ion transport in Fe and Zn contaminated water, possibly through the use of skin as a transport epithelium. More research is necessary to determine the mechanisms responsible for these trends, but overall, inanga appear to be capable of modulating ion transport in metal contaminated waters in order to prevent metal accumulation and therefore toxicity.

**Chapter 7: Cutaneous ion transport of sodium and calcium in a scaleless galaxiid;
implications for contaminant exposure**

7.1 Introduction

The skin of a freshwater fish serves as an essential barrier between the fish and its external environment, helping maintain internal ion concentrations greater than those of its surroundings. In contrast to the skin, the gills are in constant contact with the water. Additionally, branchial epithelium has a small diffusive distance, large surface area, and high rates of blood perfusion in order to achieve functions such as gas exchange (Evans et al., 2005). These attributes facilitate the diffusive loss of ions in the gills. In order to recover ions lost *via* diffusion, a freshwater fish must take up ions from the water. The gills are the principle organ responsible for this process (Evans et al., 2005); however, skin may also play an important role in ion transport (Glover et al., 2013). For example, Ca^{2+} has been shown to be actively taken up across the cleithrum skin of rainbow trout (*Oncorhynchus mykiss*) (Marshall et al., 1992). Na^+ transport has also been directly measured across the skin of the mangrove rivulus (*Kryptolebias marmoratus*) and rainbow trout (LeBlanc et al., 2010; Marshall et al., 1992). Fish exposed to extreme conditions have also been shown to use cutaneous transport as a means of overcoming compromising situations (LeBlanc et al., 2010; Nilsson et al., 2007). For example, using cutaneous Na^+ uptake to supplement gill uptake after large Na^+ losses that can occur after exercise, confinement, and handling stress (Chapter 3, Postlethwaite and McDonald, 1995) could decrease recovery times. As such, cutaneous ion transport may provide an important additional site of uptake for fish in conditions where ion transport is in jeopardy.

Inanga are an ideal candidate for studying cutaneous ion transport. Laboratory studies have shown inanga are capable of rapidly adjusting their physiology to maintain ion homeostasis, by acclimating quickly to acute changes in salinity (Chessman and Williams, 1975; Urbina et al., 2012). For instance, inanga were able to recover Na^+ balance within hours after handling stress (Chapter 3). Additionally, inanga have also been shown to use skin as a physiologically active surface in terms of both oxygen uptake and carbon dioxide excretion (Urbina et al., 2012, 2014). The long, slender body shape of inanga also provides a high cutaneous surface area to volume ratio, while the lack of scales will remove a potential barrier to ion exchange. This has been observed in the scaleless swamp eel (*Synbranchus marmoratus*) which takes up 75% of its total Na^+ requirements across the skin (Stiffler et al., 1986). In fish that utilize the skin, increased vascularization just below the surface of the skin

(Nilsson et al., 2007; Park et al., 2003) reduces diffusion distance thereby facilitating cutaneous ion exchange, providing a transport advantage over fish with scales. Inanga have also been shown to alter the transport properties of their skin through NKA isoform swapping in response to salinity changes (Urbina et al., 2013), thereby indicating a possible role for the skin in ion homeostasis.

The use of skin as a site of uptake may be especially useful in contaminated waters where ion uptake rates may be impacted. For example, contaminants like Al and Fe have been shown to result in gill clogging and damage, increasing diffusive distance and reducing available surface area for ion exchange (Dalzell and MacFarlane, 1999; Playle et al., 1989). Both of these metals are particularly important in relation to inanga as they are present at elevated levels in natural habitats (Chapters 4 & 5). If the skin is involved in ion transport, fish may be able to maintain transport rates by compensating for reduced transport at the gills. This in turn may alter their sensitivity to factors like environmental stress and trace elements. For example, trace elements have been shown to accumulate through uptake pathways of Na^+ and Ca^{2+} (Chapter 3; Hogstrand et al., 1995). Therefore, understanding ion transport across the skin of inanga may be important for understanding metal accumulation and sensitivity in this species.

In this chapter, the ability of inanga to use cutaneous ion transport was investigated using both *in vivo* (partitioning) and *in vitro* (Ussing chamber) approaches. Partitioning has previously been used to determine cutaneous ion transport in the swamp eel (Stiffler et al., 1986), allowing ion influx properties of the head and body to be independently determined. It is an important method as it preserves blood perfusion to the skin, and allows physiological control mechanisms to be maintained. It has also been successfully used in inanga to determine cutaneous oxygen uptake (Urbina et al., 2012). Calculated influx rates for the head portion, however, include the skin covering the head; so it does not completely isolate branchial from cutaneous ion transport. Using an *in vitro* technique, like the Ussing chamber, allows the isolation and examination of uptake across the skin. The Danish biologist Hans H. Ussing developed this method over 50 years ago to better understand active NaCl uptake across frog skin (Ussing and Zerahn, 1950). Since then, this technique has been used on a number of fish species to determine cutaneous ion transport (Degnan et al., 1977; Flik et al., 1996; McCormick et al., 1992). In the current study, these two techniques

(partitioning and isolated skin) were combined to investigate the cutaneous transport of Na^+ and Ca^{2+} ions in inanga using radioactive isotopes.

MRCs have long been known as the primary ion transporting cells of the gills (Foskett and Scheffey, 1982; Karnaky et al., 1977). Assuming transport mechanisms are similar between the gills and skin, the presence of these cells would also be expected in the skin. This has been shown in the skin in rainbow trout; where cutaneous Ca^{2+} transport was positively correlated with MRC density (Marshall et al., 1992). The presence of MRC in the skin of inanga would further validate the involvement of cutaneous epithelium in ion transport. There are multiple techniques for the identification of MRC in fish epithelia (Burgess et al., 1998; Ivanis et al., 2008; Nonnotte et al., 1979; van der Heijden et al., 1997). In particular, fluorescence microscopy has been shown to be a useful tool in quantifying MRC density in fish skin (LeBlanc et al., 2010; Marshall et al., 1995). As such, in the current study a fluorescent dye (MitoTracker green) was used to determine the presence of possible MRC in the skin of inanga. The presence of these cells would provide supporting evidence of the structural capacity for cutaneous ion transport in inanga.

The hypothesis being tested in these experiments was that Na^+ and Ca^{2+} transport occurs across the skin of inanga. Both partitioning and Ussing chamber experiments using radioisotopes were conducted in order to compare *in vivo* and *in vitro* techniques. Additionally, exposures to Fe, Zn, La, and verapamil were used to investigate possible mechanisms of Ca influx, and to ascertain potential impacts of metals on cutaneous uptake. Fluorescence microscopy was also used to determine the presence of possible MRC in the skin of inanga. These results will provide a better understanding of cutaneous ion transport of inanga and possible changes in susceptibility to metal contaminants.

7.2 Methods

7.2.1 Fish collection and husbandry

Fish were collected and maintained at the University of Canterbury as described in Section 2.1. All animal manipulations were approved by the Animal Ethics Committee of the University of Canterbury.

7.2.2 Partitioning

Partitioning chambers, identical to those described in Urbina et al. (2012), were constructed using Perspex with a total volume of 90 mL; 35 mL for the head portion and 55 mL for the tail. Chambers contained a sampling port through which isotope could be added, samples taken, and air flow supplied throughout the duration of the experiments. Fish (mass ranged from 0.56 to 4.81 g) were anesthetized using MS-222 (0.1 g L^{-1}) before being gently inserted through a central hole of a rubber dental dam and placed into partitioning chambers (Fig. 7.1). The rubber dental dam was stretched in order to fit the fish before being gently released to seal the head from the body. Fish within chambers were then submerged in aquarium water and left to acclimate for at least 2 hours, under constant aeration, before experiments began. Rubber bungs were then used to seal the chamber at either end before the water surrounding chambers was drained to expose the top of the chamber (and the sampling/aeration port) to air.

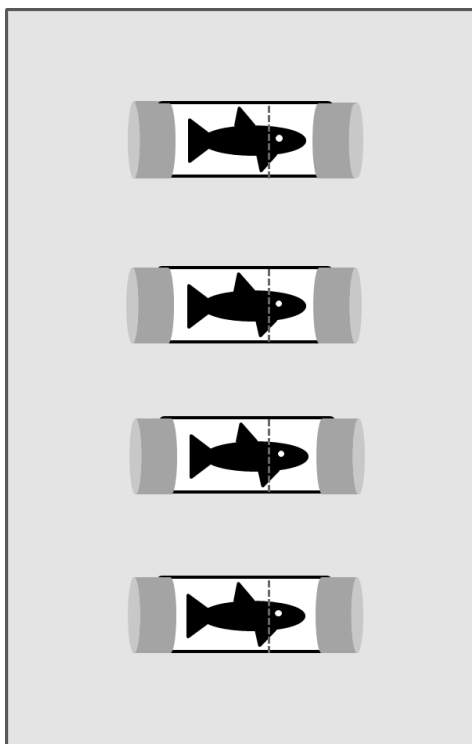


Figure 7.1 Partitioning schematic for separation of head from body. Dotted lines are rubber dental dams.

Partition chambers were constantly aerated (on both sides of the chamber) throughout the experiment, and were kept in a temperature controlled water bath at 15°C. For Na⁺ influx triplicate time zero water samples (10 mL) were taken from the water bath before fish were added to determine initial water Na⁺ concentrations (*via* flame photometry). Radiolabelled ²²Na⁺ was then added (20 µCi) to either the head or the tail portion through the sampling port and gently mixed using the pipette before triplicate 150 µL water samples were taken for specific activity calculations (Equation 2.1). After one hour, additional triplicate 150 µL water samples were taken (specific activity was calculated on the basis of averaged start and end isotope levels) and fish were removed from chambers. Fish were placed in a high Na⁺ rinse with a lethal dose of MS-222 (1 g L⁻¹) followed by two tap water rinses. The high Na⁺ rinse was used to remove any isotope adsorbed to the body surface of the fish. Spinal cords were severed before fish were weighed and whole body ²²Na⁺ was determined via a gamma counter (see Section 2.4.1). Resulting cpm were used to calculate Na⁺ influx (J_{in} ; nmol g⁻¹ h⁻¹) using Equation 2.2.

Partitioning was also conducted using ⁴⁵Ca²⁺ radioisotope to determine Ca²⁺ influx across the skin. Fish were set up and water samples taken as above, but radioactivity was analyzed *via* a liquid scintillation counter as per Section 2.10.

7.2.3 Ussing chamber

Two sections of skin, approximately 10 x 20 mm, were removed from the same fish. The portion closest to the head was termed 'head,' and the next section just below this was termed 'tail.' Muscle was removed and skin sections were independently mounted on an Ussing chamber (Warner Instruments model U-2500) insert. The model used had a 3.8 mm round opening with o-ring insets to seal around the skin. For the serosal side of the Ussing chamber, Cortland saline (124 mM NaCl, 5 mM KCl, 1 mM CaCl₂·2H₂O, 2 mM MgSO₄·7H₂O, 11.9 mM NaHCO₃, 2.9 mM NaH₂PO₄·H₂O, and 1.1 mM D-glucose) was used to act as an artificial plasma. Glucose was added to the saline before each use in order to prevent bacterial growth in stored saline. The mucosal side of the Ussing chamber was filled with aquarium freshwater (see Section 2.2 for composition). Ten mL of each solution was added. The mucosal (freshwater) side was then spiked with 10 µL ⁴⁵Ca²⁺ stock (1 µCi) and 0.5 mL

samples were taken from both sides after 5 min as time 0 and thereafter on each hour for 3 hours to determine Ca^{2+} influx (J_{ms}) across the two skin portions. Water samples were analyzed on a liquid scintillation analyzer as per Section 2.10.

Zn^{2+} has been shown to be a Ca^{2+} antagonist in rainbow trout (Hogstrand, 1995) and Zn was also one of the metals chosen for laboratory exposures in Chapter 6. As such, the use of $^{45}\text{Ca}^{2+}$ isotope to measure cutaneous Ca^{2+} transport was used in Ussing chamber experiments. By determining the effects of Zn on Ca^{2+} uptake across the skin, it can potentially provide insight into the role of the skin during exposure to Zn-contaminated water.

A similar setup, only using head portions of inanga skin, was used to determine possible effects of putative Ca^{2+} transport inhibitors on Ca^{2+} influx. Treatments included 350 μM Fe, 100 μM Zn, 1 μM LaCl_3 , and 25 μM verapamil (concentrations were similar to Rogers and Wood, 2004). Verapamil is a pharmaceutical known to block L-type Ca^{2+} channels (Li et al., 2010; Klinck and Wood, 2011). Zn is a known competitor for Ca^{2+} influx (see Section 1.4) and La is known to block Ca^{2+} channels (Weiss, 1974). Fe may cause clogging or damage leading to changes in Ca^{2+} influx (Dalzell and MacFarlane, 1999), and is a metal known to be elevated in inanga habitats (Chapters 4 & 5). The mucosal (freshwater) side was then spiked with isotope and samples collected as above. Exposures were only run for two hours as this time interval was shown, in the experiments described above, to be sufficient to detect Ca transport and in order to minimize the possible impacts of tissue degradation.

Additional experiments were run examining serosal to mucosal Ca^{2+} transport (J_{sm}). Trials with Cortland saline on both sides of the Ussing chamber were also conducted in order to determine Ca^{2+} transport under symmetric conditions. These were all run on head skin sections, with similar methodologies as above, over a three hour time period.

All water samples were analyzed on a liquid scintillation analyzer per section 2.10. Resulting cpm were then used to calculate Ca^{2+} influx according to Equations 2.1 and 2.2 (Section 2.4.1).

7.2.4 Fluorescent dye techniques

Fluorescent dye experiments followed methods similar to Esaki et al. (2009). Fish were placed in 250 mL of aquarium water with 500 nM MitoTracker green dye for

approximately 2 hours. Fish were then anesthetized using 0.1 g L^{-1} MS-222 before being placed under a confocal laser-scanning microscope (Leica TCS SP5). MitoTracker green adds a fluorescent tag to mitochondria (Evans, 2011), so was used to identify possible MRC in the skin of inanga. Pictures of the skin were taken using a 10x lens and an excitation wavelength of 530nm. Control fish that were not exposed to MitoTracker were also examined.

7.2.5 Statistical analyses

Statistical analyses followed those outlined in Section 2.11. A non-linear least squares regression analysis of Na^+ influx as a result of partitioning experiments was also run to determine the relationship between Na^+ influx and fish mass. The resulting equation and r^2 value are provided in Figure 7.5. This could not be done for Ca^{2+} , owing to the smaller number of replicates. All statistical analyses were conducted using the R software (Version 2.15.0).

7.3 Results

7.3.1 Partitioning

In vivo Ca^{2+} and Na^{+} transport occurred across both the head (gill) and tail (skin) of inanga. There was no significant difference in Na^{+} influx rates between the head and tail portions (Fig. 7.2, Student's t-test). There was, however, significantly higher Ca^{2+} influx in the head portion of inanga than in the tail (Fig. 7.3, Student's t-test, $p < 0.01$). Mean cutaneous Na^{+} influx was approximately 48% of total measured Na^{+} influx (summed average of head and tail), while cutaneous Ca^{2+} influx was only 20% of total measured Ca^{2+} influx. Using non-linear least squares regression, a polynomial line of best fit between Na^{+} influx and the mass of fish resulted in an $r^2 = 0.97$ (Fig. 7.4). There were insufficient data points to plot a similar relationship for Ca^{2+} influx.

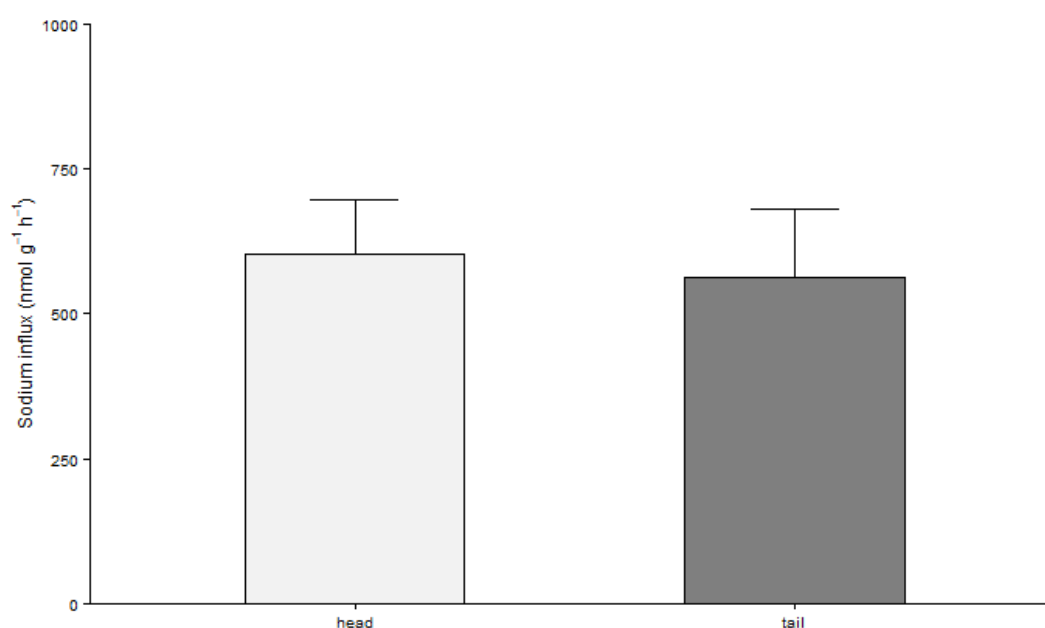


Figure 7.2 Mean Na^{+} influx ($n = 13$ head, $n = 10$ tail) ($\text{nmol g}^{-1} \text{h}^{-1}$) \pm standard error of the mean in the head and tail regions of inanga skin after *in vivo* partitioning.

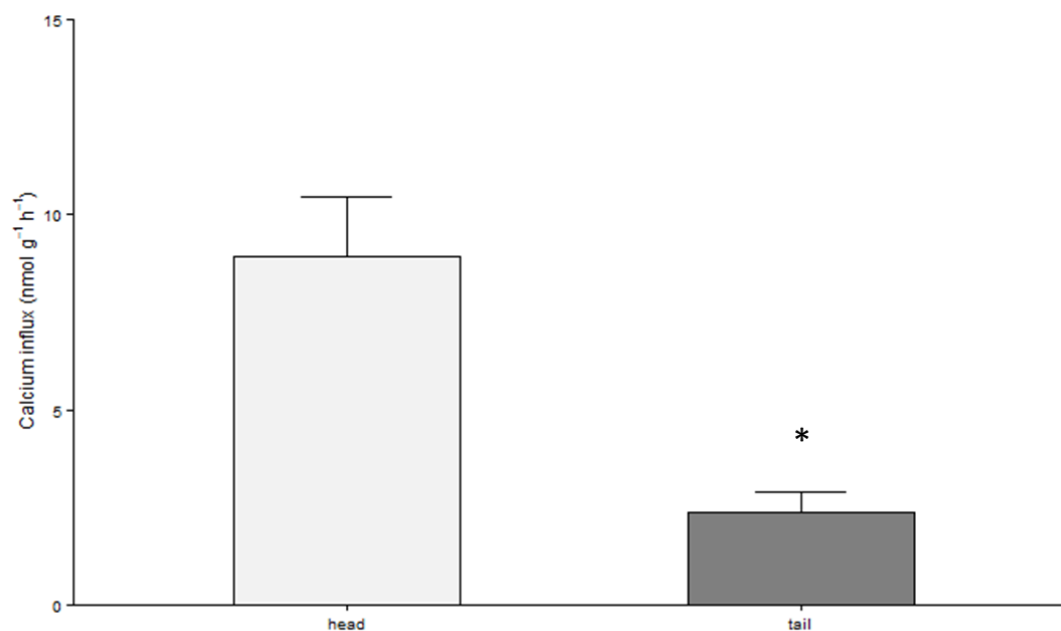


Figure 7.3 Mean Ca²⁺ influx (n = 5 head, n = 5 tail) (nmol g⁻¹ h⁻¹) ± standard error of the mean in the head and tail regions of inanga skin after *in vivo* partitioning. Asterisk (*) denotes a significant difference (Student's t-test).

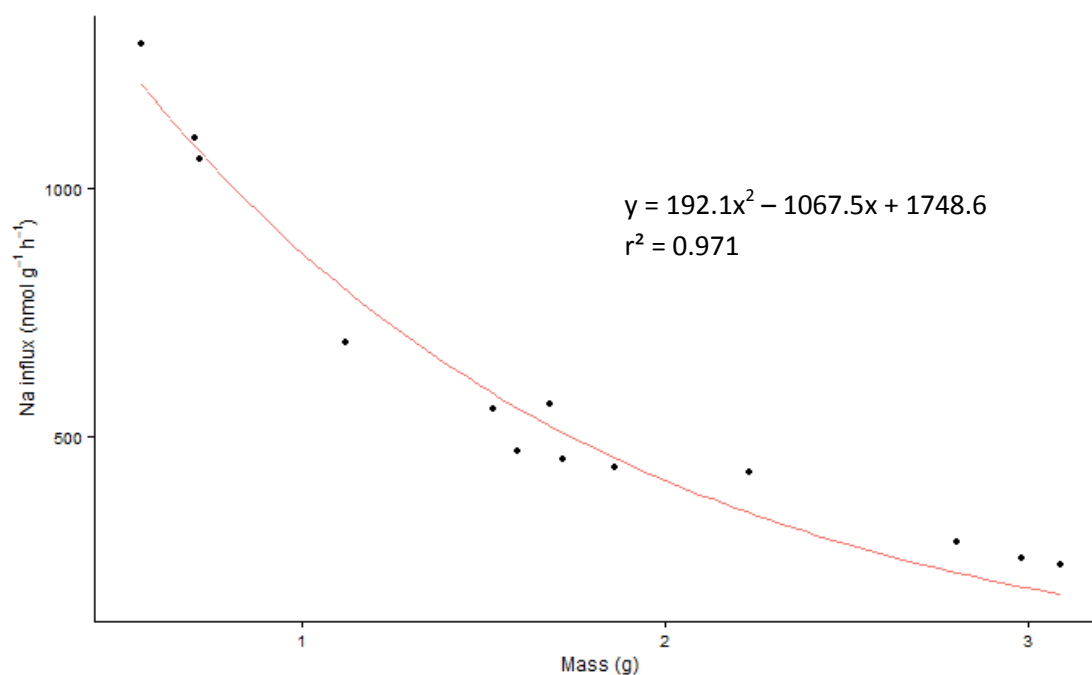


Figure 7.4 Non-linear least squares regression for Na⁺ influx (nmol g⁻¹ h⁻¹) in the head portion of inanga (n = 23) as it relates to total fish mass (g) after *in vivo* partitioning.

7.3.2 Ussing chamber

Differences in *in vitro* Ca^{2+} influx between the head and tail portions were just below statistical significance (Fig. 7.5; one-way ANOVA, $p = 0.06$). Control fish ($n = 2$) had significantly higher Ca^{2+} influx rates than those with inhibitors (Fe, La, verapamil, and Zn) (one-way ANOVA, Fig. 7.6, $n = 4$ each). Ca^{2+} influx for Zn treatments was also significantly higher than those with La and verapamil treatments (one-way ANOVA, $p = 0.026$ and $p = 0.033$, respectively).

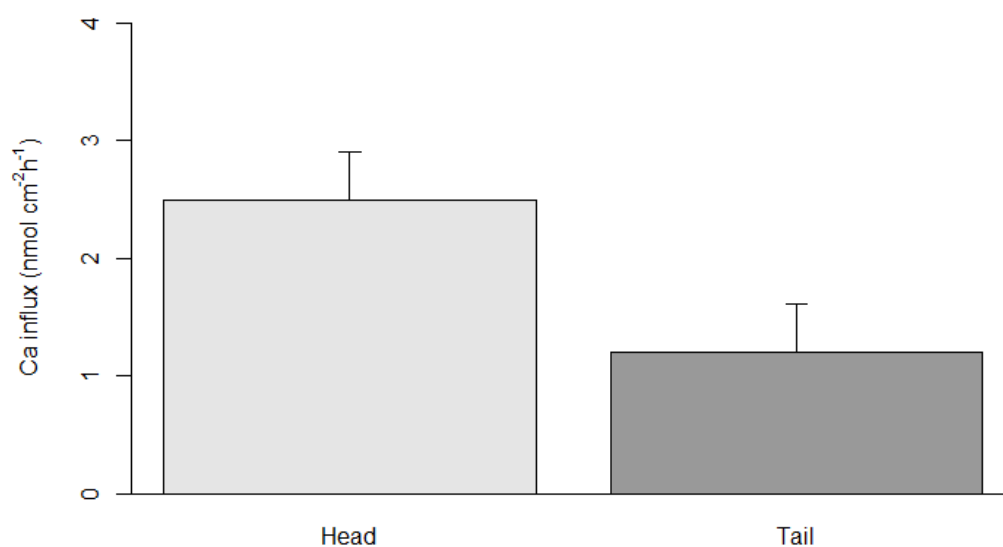


Figure 7.5 Mean Ca^{2+} influx (J_{ms} , $\text{nmol cm}^{-2} \text{h}^{-1}$) \pm standard error of the mean in the head ($n = 18$) and tail ($n = 17$) portion of inanga skin after *in vitro* Ussing chamber experiments.

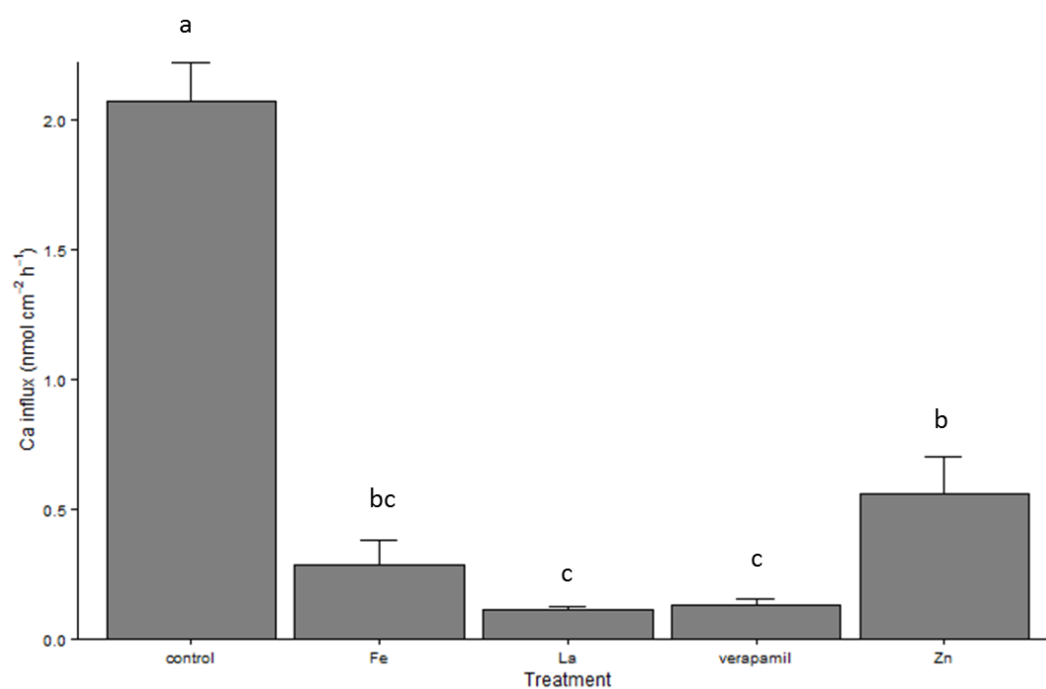


Figure 7.6 Mean Ca²⁺ influx (J_{ms}, nmol cm⁻² h⁻¹) ± standard error of the mean in the head portion of inanga skin as controls (n = 2), and after treatments (n = 4 each) of Fe (350 μM), Zn (100 μM), La (1 μM), and verapamil (25 μM) using an *in vitro* Ussing chamber technique. Bars sharing letters do not differ significantly in Ca²⁺ influx rates (one-way ANOVA).

There was significantly higher serosal to mucosal (J_{sm}) Ca^{2+} transport across the skin than mucosal to serosal (J_{ms}) when transport of both head and tail portions of skin were combined (Fig. 7.7; one-way ANOVA, $p < 0.01$). Transport rates were combined, as separately these two sections did not differ in transport rates (data not shown). The resulting calculated flux ratio (J_{ms}/J_{sm}) was 0.29. Trials using symmetric conditions (Cortland on both sides) showed no ion transport (mean = $0.01 \text{ nmol cm}^{-2} \text{ h}^{-1}$, data not shown).

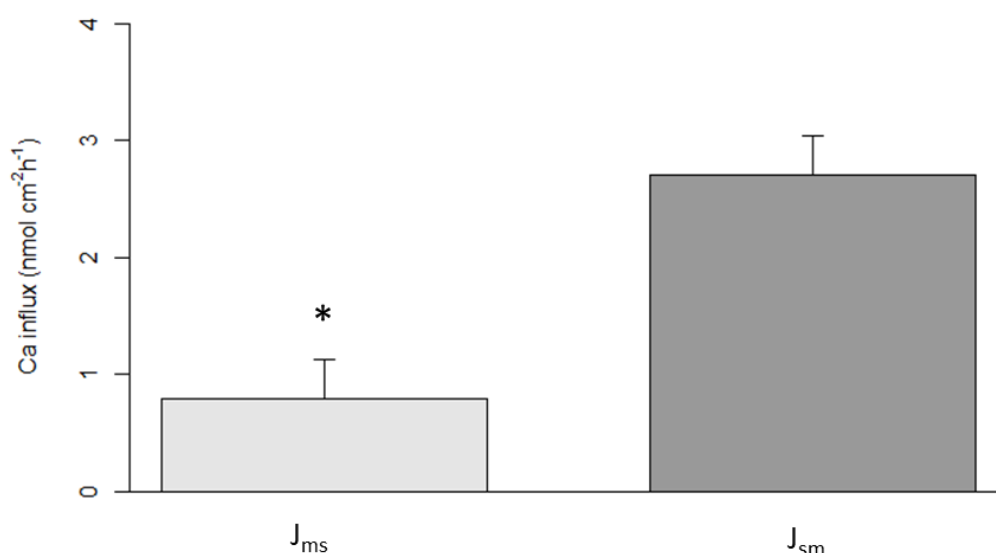


Figure 7.7 Mean Ca^{2+} influx (J_{ms} , $\text{nmol cm}^{-2} \text{ h}^{-1}$, $n = 17$) and Ca^{2+} efflux (J_{sm} , $\text{nmol cm}^{-2} \text{ h}^{-1}$, $n = 18$) \pm standard error of the mean in combined head and tail portions of inanga skin. Asterisk denotes a significant difference between influx rates as determined by a one-way ANOVA.

7.3.3 Fluorescent dye

Control fish showed reflective star-shaped cells across the whole body at a wavelength of 530nm (Fig. 7.8). Fish exposed to Mitotracker green dye showed cells high in mitochondria in cutaneous epithelia across the whole body; although at a higher density close to the head (Fig. 7.9).

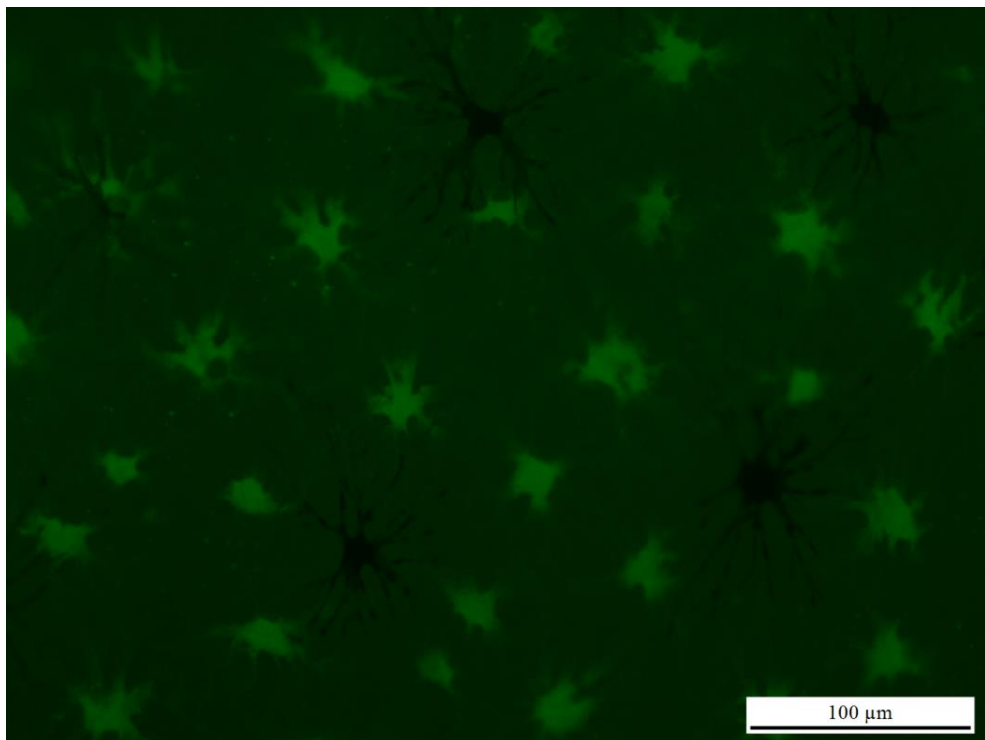


Figure 7.8 Image of the skin of inanga posterior to the head for control fish (no dye) with reflective star-shaped cells. The image was captured using a confocal laser-scanning microscope with a 10x lens and an excitation wavelength of 530nm.

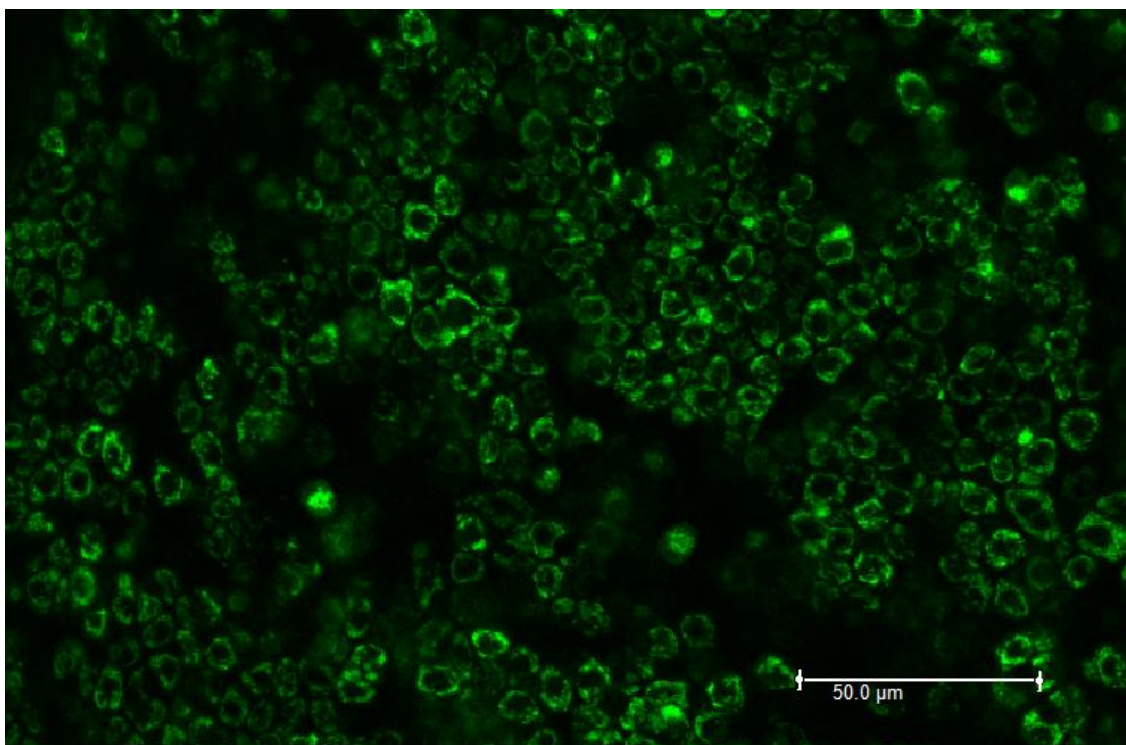


Figure 7.9 Image of the skin of inanga posterior to the head after being exposed to Mitotracker green dye (1 mM) for 2 hours (green cells are putative MRC). The image was captured using a confocal laser-scanning microscope with a 10x lens and an excitation wavelength of 530nm.

7.4 Discussion

The results of this study clearly indicate that inanga are capable of transporting both Ca^{2+} and Na^{+} across the skin. Na^{+} influx in the skin was nearly equivalent to that in the head portion (Fig. 7.2), suggesting up to 50% of Na^{+} influx occurs in the skin of inanga. This is one of the highest cutaneous Na^{+} influx values on record (Glover et al., 2013). Cutaneous Na^{+} influx represented a greater proportion of total Na^{+} uptake than the proportion of cutaneous Ca^{2+} influx to total Ca^{2+} influx. Additionally, total Ca^{2+} influx rates in inanga were lower than those in the killifish and rainbow trout (Prodocimo et al., 2007). The proportion of cutaneous Na^{+} uptake highlights the importance of this additional uptake site in inanga ion transport physiology.

7.4.1 Cutaneous Na^+ transport

The higher relative magnitude of cutaneous Na^+ uptake in comparison to Ca^{2+} uptake *in vivo* is likely due to higher internal Na^+ demands; suggesting cutaneous Na^+ influx may be more important than Ca^{2+} influx in inanga. This may, in part, be due to the important role Na^+ plays in homeostasis, including ammonia excretion and acid/base balance (Evans et al., 2005). In order to maintain internal Na^+ levels, Na^+ influx must be constantly maintained (Evans et al., 2005). This difference in relative uptake between Na^+ and Ca^{2+} may also be an important adaption to changes in salinities. As inanga are amphidromous (McDowall, 1989), they are faced with large changes in salinity. The ability to use the skin as an additional site of Na^+ uptake may be important when migrating from the sea to freshwater streams, where large Na^+ losses would be expected (Talbot and Potts, 1989). Exposures at different salinities, acidity, and water hardness would help identify which of these transport mechanisms are utilized in cutaneous ion transport. Additional molecular analyses would also help determine specific transporters (Scott et al., 2004; Yan et al., 2007).

Partitioning is one of the only available *in vivo* techniques for analyzing ion transport across the gill *versus* skin epithelium in an intact fish. This method allows blood perfusion of the skin to be maintained; however, it may result in a significant amount of stress on the fish, owing to significant handling. Fish in the current study were left for two hours after being placed in partition chambers in order to recover from handling stress (Chapter 3). The Na^+ influx rates in this study (about $600 \text{ nmol g}^{-1} \text{ h}^{-1}$) are similar to those of unstressed fish in Chapter 3 (about $500 \text{ nmol g}^{-1} \text{ h}^{-1}$), suggesting inanga were able to recover from setup stressors. These rates were also similar to tamoata (*Hoplosternum littorale*) (Baldisserotto et al., 2012) and rainbow trout (Matsuo et al., 2004) under control conditions, suggesting these are resting Na^+ influx rates.

7.4.2 Cutaneous Ca^{2+} transport

Ca^{2+} influx in the gill is achieved most often *via* facilitated diffusion through Ca^{2+} channels (ECaC or equivalent) driven by a concentration gradient created by low cellular Ca^{2+} concentrations (Flik and Verbost, 1993; Hwang and Lee, 2007). After Ca^{2+} enters the

cell, it is then transported to the blood either through a basal Ca^{2+} -ATPase (which is responsible for lowering intracellular Ca^{2+}), or exchanged for Na^+ *via* an NCX transporter facilitated by the Na^+ gradient produced by NKA (Dymowska et al., 2012; Hwang and Lee, 2007). Partitioning studies showed that although the head takes up the majority of Ca^{2+} ($8.9 \text{ nmol g}^{-1} \text{ h}^{-1}$), the skin also made a significant contribution ($2.4 \text{ nmol g}^{-1} \text{ h}^{-1}$). The ability of the skin to transport Ca^{2+} was also confirmed *in vitro*. Ussing chamber experiments found that the proportion of cutaneous Ca^{2+} influx was significantly lower in portions of the skin closer to the tail than those near the head (Fig. 7.5). The use of known inhibitors (LaCl_3 and verapamil) in this setup also revealed a significant inhibition of cutaneous Ca^{2+} influx. This suggests transporters in the skin are similar to those in the gill. Specifically, LaCl_3 is known to block T-type Ca^{2+} transporters (Weiss, 1974), while verapamil (a prescribed pharmaceutical that can be found in aquatic environments) is known to block L-type Ca^{2+} channels in the gill (Li et al., 2010) (Fig. 7.6). Because both of these inhibitors blocked Ca^{2+} uptake in inanga skin, it suggests both T- and L-type Ca^{2+} channels are present.

In freshwater trout (*Salmo gairdneri*) Ca^{2+} efflux has been shown to be a passive, paracellular process dependent on concentration gradients (Perry and Flik, 1988). However, the results of Ussing chamber experiments using known inhibitors suggest cutaneous Ca^{2+} transport may also be an active process in inanga. The resulting flux ratio ($J_{\text{ms}}/J_{\text{sm}}$) of 0.29 in the current study was similar to calculated flux rates across the cleithrum skin of rainbow trout (0.26), which used similar serosal/freshwater bathing solutions to those in this study (Marshall et al., 1992). This flux ratio means that the rate of Ca^{2+} efflux is nearly 4 times higher than influx. The flux ratio from the Marshall et al. (1992) study was higher than the predicted Ussing flux ratio (0.08), indicating this ion transport was active. Although the Ussing flux ratio would need to be calculated for the current study, it does infer that this Ca^{2+} transport in inanga skin is also active.

The lack of Ca^{2+} transport in symmetric conditions may have been a result of the disappearance of a transepithelial potential (TEP). TEP is the voltage across the epithelium, or the sum of both apical and basolateral membrane potentials (Potts, 1984). A disappearance of TEP was also shown in goldfish under symmetric conditions (Eddy, 1975). TEP is necessary for ion transport as it drives electrogenic ion transport (Claiborne and Evans, 1984). Higher concentration of cations in Cortland saline when compared to

freshwater may have also prevented Ca^{2+} influx (Potts, 1984) through changes in TEP, leading to no net movement of Ca^{2+} .

7.4.3 The importance of mucus

In asymmetric flux conditions (mucosal freshwater/serosal Cortland saline), which represents the natural setting of the skin, a large Ca^{2+} efflux (J_{sm}) was observed (Fig. 7.7). Similar findings have been observed in rainbow trout isolated skin preparations (Marshall et al., 1992). This condition (larger Ca^{2+} efflux than Ca^{2+} influx) may have been due to the removal of body mucus during sample preparation. As a scaleless fish, inanga are instead considered to rely on a coating of body mucus to act as a barrier between the fish and the environment (McDowall, 2010). It is possible that the presence of mucus on the external surface of fish skin affects Ca^{2+} transport. The presence of this mucus microenvironment, containing multiple polyanionic binding sites (Shephard, 1994), may have trapped Ca^{2+} ions as they were excreted. Furthermore, mucus may have also been able to bind Ca^{2+} in the water, concentrating it against the skin surface. The acidification of the mucus layer would result in higher concentrations of protons that could out-compete Ca^{2+} for binding sites and cause Ca^{2+} to become available for transport back into the cell. During skin preparation for Ussing analysis, the mucus layer covering the skin was likely removed, thus potentially removing a mechanism that would have facilitated a higher Ca^{2+} influx, and reduced Ca^{2+} efflux.

The mucus of fish has a range of roles; including immunity, osmoregulation, defense, feeding, respiration, and other species-specific functions (Shephard, 1994). The role of mucus will vary according to its biophysical properties, epithelial type, and life history traits of the fish (Shephard, 1994). While the specific function of mucus in terms of ion transport is still unclear, it is possible that the removal of mucus from the inanga skin during sample preparation may have led to changes in ion transport. Although Ca^{2+} diffusion rates in the gills of rainbow trout were unaffected by the presence of mucus (Pärt and Lock, 1983), intestinal mucus has been shown to be an important component in intestinal metal uptake (Glover and Hogstrand, 2002b). For example, the mucus in the intestine is thought to aid in transport by trapping cations and generating high concentrations at the uptake surface

(Glover and Hogstrand, 2002b). This ability to trap cations may have served an important role in concentrating Ca^{2+} ions at the site of uptake. It should also be noted that experimental fish from the Pärt and Lock study (1983) were treated with formalin in order to remove the mucus, and formalin has been shown to impair the ability of the gills to maintain osmotic and acid/base balance (Wedemeyer, 1971).

7.4.4 Scaling effects

The Na^+ influx rates for inanga in this study are consistent with those expected for a fish of their size, according to the scaling relationship between fish mass and Na^+ uptake proposed by Grosell et al. (2002). Similar to Grosell et al. (2002), mass-specific Na^+ influx rates in inanga decreased as mass increased. The non-linear regression for Na^+ influx (for the head region) as it relates to body mass also produced a curve with a high proportion of variance in Na^+ influx having been explained by fish size (Fig. 7.4, $r^2 = 0.971$), supporting the idea that Na^+ turnover rates scale with mass. As diffusive Na^+ loss is higher in organisms with a higher surface area to mass ratio, Na^+ uptake requirements would also be higher (Grosell et al., 2002). This higher Na^+ turnover rate of smaller fish typically makes them more susceptible to contaminants that affect Na^+ transport (Grosell et al., 2002). This is either due to a higher number of transporters allowing more of the contaminant to enter, or a higher transport affinity, meaning effects will be more deleterious. As a small fish with high Na^+ turnover rates, it is predicted that inanga may be more sensitive to contaminants. However, this is contrary to the findings of Chapters 4-6, as inanga appeared relatively resilient in the face of elevated trace elements. This suggests that inanga are only sensitive to certain trace elements. For example, neither exposure metal in Chapter 6 was a Na^+ mimic. Zn is a Ca^{2+} mimic, but as Ca^{2+} exhibited a lower proportion of cutaneous influx rates, inanga may be less sensitive to this type of contaminant. The Na^+ mimic (Cu) used in Chapter 3, however, did show significant accumulation. The lack of accumulation and significant changes in biomarkers in caging studies (Chapters 4 & 5) may have been due to low levels of Na^+ mimics or the presence of NOM and other trace elements.

7.4.5 Metal toxicity

As Fe was one of the contaminants found to significantly accumulate (or nearly so) in both caging studies (Chapters 4 & 5), it was used in Ussing trials to determine its impact on Ca^{2+} influx across the skin of inanga. The addition of 350 μM Fe to the mucosal side of the Ussing set-up resulted in a significant decrease in Ca^{2+} influx (Fig. 7.6). It is also possible that Fe impaired Ca^{2+} influx in a more specific manner. Fe^{2+} has been shown to block both L-type (Oudit et al., 2003) and T-type (Lopin et al., 2012) Ca^{2+} channels in mammalian cardiomyocytes. These channels are thought to be similar to those in freshwater fish (Dymowska et al., 2012), and as Fe exposure showed significant decreases in Ca^{2+} influx, it is likely that one or both of these transporters are present in the skin of inanga. Furthermore, oxidative stress as a result of Fe-induced products from the Fenton reaction (Equation 1.1) have also been shown to inhibit Ca^{2+} -ATPase, preventing Ca^{2+} transport (Moreau et al., 1998).

Toxicity in freshwater fish due to elevated Zn concentrations is thought to be a result of hypocalcemia caused by both impaired Ca^{2+} influx through competitive inhibition, and the inhibition of the basolateral Ca^{2+} -ATPase (Hogstrand et al., 1994; Hogstrand and Wood, 1996; Spry and Wood, 1985). The addition of 100 μM Zn to mucosal solutions in Ussing trials resulted in a significant decrease in Ca^{2+} influx (Fig. 7.6), suggesting a similar Ca^{2+} competition and inhibition in the skin. If Zn is acting as a competitive inhibitor, increasing Ca^{2+} concentrations should then ameliorate Zn toxicity. This ameliorative effect of increased water hardness has previously been shown to reduce Zn^{2+} , Cd^{2+} , and Cu^{2+} toxicity (Hogstrand et al., 1995; Verbost et al., 1997). Trials investigating how varying concentrations of Ca^{2+} affect this inhibition could help reveal the extent to which cutaneous ion transport is affected by Zn exposure in inanga.

7.4.6 Mitochondria-rich cells

MRC's are the main ion regulatory cells in freshwater fish gills (Evans, 2011; Wood and Pärt, 1997). They are responsible for the uptake and excretion of a number of ions and other physiologically relevant molecules (Evans, 2011). Although uptake in cutaneous

epithelia may vary from that in branchial epithelia, it is likely MRC would need to be present in the skin in order for cutaneous ion transport to occur. This is supported by correlations between MRC density and Ca^{2+} influx in both the gills and cleithrum skin of rainbow trout (Marshall et al., 1992; Perry and Wood, 1985). Supporting this, histological studies of swamp eel skin showed similar structures in the cutaneous epithelium to those in the gill (Stiffler et al., 1986). Without evidence of these cells, it would be difficult to argue that cutaneous ion transport occurs. MitoTracker green results showed cells with high numbers of mitochondria in the cutaneous epithelia of inanga (green cells in Fig. 7.9). The presence of these cells suggests the presence of MRC thereby supporting the hypothesis that inanga are capable of cutaneous ion transport. Additional dyes (DASPEI, DAPI, etc.), further microscopy (scanning electron microscopy), and immunohistochemical staining (Carmona et al., 2004; Fernandes et al., 2013) could all help to more conclusively identify these cells as MRC.

7.4.7 Chromatophores

During confocal microscopy, fish unexposed to dye showed reflective, star-shaped cells in the cutaneous epithelium (Fig 7.8). The origin of the genus name for inanga (*Galaxias*) is due to the spotted pattern of the skin and its resemblance to a galaxy (McDowall, 1989). The cells responsible for these spots are dermal chromatophores (Elliot, 2000). These are classified into five groups depending on color and reflectivity; and because of their shared ontogenetic origin with neurons, most are dendritic cells with branches or extensions (Elliot, 2000). Chromatophores with reflective properties can be categorized as one of two types: leucophores and iridophores (Iga and Matsuno, 1986). As leucophores and iridophores use different organelles to exert this reflectivity (Iga and Matsuno, 1986), determining the contents of these cells would be necessary to elucidate which chromatophore is present in inanga skin. These cells may be important in either sexual selection, predator avoidance, or both (Elliot, 2000; Harper and Case, 1999; Ikejima et al., 2008). Conclusions regarding their function, however, would require more research and is beyond the scope of this study; as they are unlikely to play a role in ion transport.

Chapter 8: General discussion

8.1 Ion transport in inanga

This thesis provided new knowledge on the mechanisms of ion transport in inanga. Results of Chapter 3 revealed stress to cause large disruptions to Na^+ flux rates; however inanga were able to rapidly recover from this ionoregulatory stress. Additionally, the skin of inanga was shown to be a significant site of Ca^{2+} and Na^+ uptake. This is the first study to reveal these ion transport capabilities in inanga.

The results of Chapter 3 showed a significant increase in Na^+ efflux as a result of handling stress. This efflux was subsequently matched with a significant increase in Na^+ influx, with rates nearly recovering in as little as two hours and fully recovering after 24 hours. This large compensatory Na^+ response reveals the capacity of inanga to rapidly change ion transport rates, confirming their ionoregulatory plasticity (Chessman and Williams, 1975). This capacity to alter transport rates was also seen in inanga through rapid transporter isoform switching due to salinity changes (Urbina et al., 2013).

Chapter 7 revealed that cutaneous Na^+ influx occurred at a much higher relative proportion of ion uptake than cutaneous Ca^{2+} influx. Na^+ influx was also shown to scale with body size, similar to those of other freshwater fish (Grosell et al., 2002). The higher surface area to mass ratio of juvenile inanga suggests they would need to compensate for higher rates of efflux. As such, cutaneous uptake could perform an important role in achieving ion balance. Furthermore, ventilation frequency was also shown to correlate to Na^+ efflux rates ($r^2 = 0.8706$, Fig. 3.5). Increased ventilation during exercise can lead to Na^+ loss (Chapter 3) which may then result in the skin being used in a compensatory manner. Cutaneous Na^+ influx may be an important adaption to changes in salinities, as inanga are amphidromous and juveniles need to be able to cope with both freshwater and saltwater within a short time-frame. Adults, however, do not face such drastic changes in salinity, so a reduction in this ion transporting capability might be expected as the fish ages.

Both partitioning and Ussing chamber experiments in Chapter 7 revealed a significant amount of ion transport occurred across the skin of inanga (up to 48% of total measured influx). This is among the highest measured cutaneous ion uptake rates in freshwater fish (Glover et al., 2013). This cutaneous ion transport should allow skin to be used in a significant ionoregulatory capacity. The lack of scales in inanga is likely an important reason for the significant use of cutaneous ion transport in this species. As such,

the role of the skin in inanga functions as both a barrier minimizing diffusive loss, and as a site of ion transport.

8.2 Metal toxicity

As mentioned above, exercise stress (i.e. migration) can result in a large Na^+ efflux. The results from Chapter 3 showed that Cu uptake significantly increased as a result of the subsequent increase in Na^+ influx. These results, combined with the high Na^+ uptake rates related to size, suggest inanga are more susceptible to Cu toxicity during exercise stress. This susceptibility is also likely exacerbated by the fact that inanga migrate as juveniles, who would have even higher Na^+ turnover rates, relative to adult fish. This would potentially exacerbate the known higher sensitivity of early life-stages of aquatic species to metal toxicants (Bianchini et al., 2002). This enhanced sensitivity to Cu appears to contrast results of Chapters 4-6, which only showed increases in a few toxic endpoints. However, as mentioned in Section 7.4.4, this is likely due to the variable sensitivity of inanga to certain trace elements. With lower Ca^{2+} turnover rates than killifish and rainbow trout (Prodocimo et al., 2007), inanga would likely be less sensitive to Zn toxicity, and dissolved Fe may not have been high enough to cause toxicity.

The addition of both Fe and Zn in Ussing chamber experiments (Chapter 7) resulted in the inhibition of cutaneous Ca^{2+} influx. This suggests that the uptake of ions via the skin may also be impacted by metal contaminants in a manner similar to the gills of other fish (Hogstrand et al., 1994; Peuranen et al., 1994). However, *in vivo* exposures showed no significant change in Ca^{2+} influx (Chapter 6). This may be due to physiological controls within the fish that were removed when examined *in vitro* (i.e. hormones). For example, hormones such as cortisol have been shown to directly modify ion transporters responsible for Na^+ handling at the gills in rainbow trout (*Oncorhynchus mykiss*) (Ivanis et al., 2008). However, *in vitro*, none of these control mechanisms would be available. As such, the skin may only be capable of compensating for contaminant effects with certain exposures. Including hormone measurements *in vivo* and the addition of specific hormones *in vitro* could help elucidate their effect on cutaneous ion transport in inanga. This could then help determine whether or not inanga are capable of adjusting ion transport in the face of Fe and Zn contamination.

Although these results appear to contrast one another, *in vivo* results suggest a relative tolerance to Fe and Zn contaminated water. While *in vitro* evidence suggests these metals are capable of disrupting Ca^{2+} transport, it is likely inanga are capable of overcoming these effects.

The results of the two *in situ* caging studies (Chapters 4 & 5) revealed a relative resilience of inanga in the face of elevated trace elements. Both studies showed a significant increase in Al accumulation, while Fe accumulation was only significantly higher in Chapter 4 (although it fell just below significance in Chapter 5). Aside from one stream at one time point, there were no significant increases in measured biomarkers in the second study (Chapter 5), suggesting inanga were able to withstand elevated trace elements without excessive LPO or impacts on NKA activity. The singular increase in TBARS at LC in Chapter 5 was likely due to pH stress, or changes in Al bioavailability as a result of pH. Both studies support the idea that diet may be playing a role in trace metal burdens, as water and sediment trace element concentrations did not correlate to whole body accumulation. Dietary uptake has also been seen in a similar transplant study where increases of Cu in the gut were attributed to dietary exposure in yellow perch (*Perca flavescens*) (Kraemer et al., 2005a). Both Al and Fe are thought to be respiratory toxicants that tend to accumulate on the gills (Dalzell and MacFarlane, 1999; Gensemer and Playle, 1999). However, only Al was significantly different between whole and incomplete body samples in Chapter 4 (Fig. 4.2a), suggesting accumulation on the gill or GI tract. Instead, Fe accumulation between whole and incomplete body sample types was not significantly different, even in streams where Fe concentrations were significantly higher than the source. This supports the hypothesis that these elements may have been accumulated *via* the diet. As Fe is a nutritionally required element, it would be expected to be readily absorbed in the GI (Bury et al., 2012); whereas Al is not essential (Wilson, 2012), so would not be taken up as effectively, leading to accumulation on the surface of the GI tract.

Subsequent laboratory Fe and Zn exposures with inanga (Chapter 6) revealed little to no short-term changes in whole body metal burden, even with trace element concentrations higher than those in the field. There were also no statistically significant differences in ammonia production, ion influx rates, or biochemical biomarker (TBARS). Chronic exposures to Zn in rainbow trout also showed little to no accumulation, suggesting this nutritionally required trace element may be relatively well regulated (McGeer et al.,

2000a). However, Fe exposures have shown to accumulate in different tissues, based on the type of Fe used and the NOM levels in the water (Lappivaara et al., 1995). As such, different Fe exposure criteria may lead to significant accumulation. Fe exposure did, however, lead to significant changes in respiration, suggesting Fe may have accumulated on the gills; which were removed for biochemical assays. It is possible that these trace elements are being sequestered in specific organs, leading to subsequent physiological costs. For example, measuring excretion rates using radiotracers could have helped determine whether uptake was prevented or simply excreted before measurements.

8.3 Methodological considerations

Field studies were limited in that accumulation was measured in fish with organs removed. This left a gap in terms of tissue-specific accumulation. As the removed organs of inanga are very small, additional analyses, such as trace element concentrations could not be performed. This would have been useful in determining whether Al and Fe had accumulated on, and clogged, the gills, as they are both prone to do (Dalzell and MacFarlane, 1999; Playle et al., 1989). However, these organs were removed in order to have more than one measurement of toxicity, which is important as accumulation does not explain specific physiological impacts of exposure.

Both laboratory and *in situ* caging studies revealed the complexities in terms of predicting trace element accumulation in inanga. Models based on water quality parameters alone did not appear to be sufficient for predicting how trace elements will accumulate in inanga. The results of both Chapters 4 and 5 confirm the need for field studies when examining responses to trace elements. They allow for realistic exposures including variables such as exposure to prey, natural flow, and other variables, which are often unaccounted for in the laboratory. However, these field studies are very difficult and unexpected problems (such as inclement weather) can arise. Using the combination of both laboratory and field studies is a good way to balance the pros and cons of each setting, and learn more about specific mechanisms of metal uptake and toxicity.

The recovery from handling stress in Chapter 3 clearly supports the need for appropriate acclimation periods before running experiments, as this ion loss can

significantly affect the physiology of the fish. It also revealed that although inanga are a schooling fish, the use of individual fish does not lead to significantly different results. This is good from an ethical and logistical viewpoint, as using individual fish as opposed to schools of fish allows for a smaller number of experimental animals, and makes animal collection, care, and setup far easier. This is an important finding for future inanga research, as an unnecessary number of animals will not have to be manipulated.

8.4 Environmental perspectives

The results of Chapter 3 suggest that other stressors, such as the exercise stress experienced during migrations, could also lead to large Na^+ efflux. This would subsequently make inanga more at risk to disruption in ion balance. It would also result in higher Cu accumulation should migratory streams have elevated levels of Cu. Furthermore, the near-coastal waters where these migrations occur are also vulnerable to other forms of anthropogenic pollution (e.g. Greig et al., 2010). Na^+ challenges that result from exercise stress may further threaten inanga in the face of additional ion disrupting pollutants. For example, polycyclic aromatic hydrocarbons and organochlorides have been found to cause gill lesions which could further challenge osmoregulation (Oliveira Ribeiro et al., 2005).

The ability of inanga to use cutaneous ion transport may be a double-edged sword; conferring an advantage in extreme environments, but also making them more susceptible to contaminants that specifically affect ion transport. For example, in Chapter 7, both Ca^{2+} and Na^+ were shown to be taken up across the skin, so in low Ca^{2+} or Na^+ waters, the skin may act as an additional uptake route, increasing total uptake. However, both Fe and Zn were shown to block this transport in Ussing chamber experiments, suggesting the skin may, instead, be an additional site of accumulation/toxicity in metal contaminated waters.

8.5 Regulatory context

The aim of this study was to assess trace element toxicity and its effects on ion transport in an environmentally relevant context so results could be related to regulatory considerations regarding inanga. For example, the results of Chapter 3 suggest stress can lead to an increase in Cu uptake, exacerbating toxicity. As total body Cu burden has been shown to relate to toxicity in a dose-dependent manner (Santore et al., 2001), increased Cu absorption will lead to increased toxicity. This is a concern as fish in more disruptive environments may not be adequately protected if limits are based on relationships between accumulation and toxicity based on unstressed fish. Stress due to rod and line fishing practices has shown to result in elevated blood cortisol in carp (*Cyprinus carpio*) (Pottinger, 1998) and snapper (*Pagrus auratus*) (Pankhurst and Sharples, 1992). Although inanga are typically caught with seine nets, it is likely the stress, and therefore cortisol release, associated with rod and line fishing applies to seine netting as well. As cortisol has been shown to result in Na⁺ efflux, it is likely that fishing stress would also lead to increased Cu uptake. Consequently, exercise stress during migrations and stress due to fishing should be important considerations when implementing management strategies.

The results of this research suggest the BLM approach is one that requires further validation in terms of its applicability to inanga. Very few of the trace elements showed a significant correlation between water or sediment concentrations and the resulting body burden. Additionally, laboratory exposures (Chapter 6) showed no accumulation and very few changes to physiological and biochemical endpoints, suggesting little to no toxic impacts at relatively high concentrations. Although Al and Fe accumulated in some caged streams (Chapters 4 & 5), biomarkers did not change according to exposure levels (increases were only found in the lowest and highest Fe exposure groups). As such, the typical BLM relationship may not to apply. There are likely factors the BLM does not take into account that are affecting accumulation and toxicity in inanga. For example, dietary exposure, which is not accounted for in the BLM can be a significant portion of exposure to fish (Clearwater et al., 2002). It can also play an ameliorative effect, as dietary Na⁺ was shown to have on Cu accumulation in rainbow trout (Kjoss et al., 2005).

Current trigger values in New Zealand for protecting aquatic biota from Al toxicity do appear to be low enough to protect inanga. These values also account for changes in Al

bioavailability as a result of pH, which was shown to be an important variable in Chapter 5, with significant correlations between pH and measures of toxicity (whole body accumulation and TBARS). However, 18 out of the 34 streams surveyed (53%) in Chapter 3 had Al concentrations above this trigger value ($0.8 \mu\text{g L}^{-1}$ Al, $\text{pH} < 6.5$) (Appendix 2). As such, a priority should be placed on the implementation of these criteria, i.e. monitoring and restoration, before determining whether a more accurate trigger value is necessary.

8.6 Future research

Building on the studies in this thesis would help better elucidate the specific mechanisms behind measured responses. For example, measuring ion transport after exercise stress, would help reveal whether inanga suffer from ion disruption during migration. Additional research comparing cutaneous ion transport in juveniles and other land-locked galaxiids to those of adults in this study may also help elucidate whether cutaneous ion transport is an adaptation to migratory patterns. Furthermore, using a range of waters with varying Ca^{2+} and Na^{+} concentrations could help elucidate the extent to which inanga use cutaneous ion transport in a compensatory manner. If the skin is being used to compensate for uptake, relative levels of cutaneous uptake would be expected to increase in low Ca^{2+} and Na^{+} waters. Additional partitioning experiments measuring plasma cortisol and prolactin levels in Fe and Zn contaminated waters, followed by Ussing chamber experiments with varying levels of hormones to measure specific effects on transporters could also help determine whether the control of cutaneous ion transport is hormonal, and whether this control can lead to reduced metal uptake. Additional biomarkers (such as GSH, GST, cortisol, and protein carbonyls), effects on swimming speed, and possible behavioral effects could also help determine the costs associated with preventing accumulation in trace element exposures. Additionally, more specific laboratory exposures with varying concentrations of DOM, pH, and water hardness would help confirm whether these variables have similar effects on metal accumulation in inanga as fish for which the models are based; and as such whether or not the BLM is sufficient in predicting toxicity to inanga.

In order to better understand the mechanisms behind trace element toxicity to inanga, exposures at higher metal concentrations, varying water chemistries, and more

biochemical endpoints are necessary. Specifically, exposures to Al under varying pH and dietary Al concentrations would help tease apart the impacts each has on Al accumulation. For example, inanga have been shown to inhabit acidic streams (Olsson et al., 2006) which would then have higher amounts of bioavailable Al than neutral streams. Additional exposures under varying pH are necessary to determine whether this adaptation to acidic waters makes inanga more or less susceptible to Al toxicity. As inanga showed high Na^+ flux rates, exposures to trace elements that compete with Na^+ (i.e. Ag and Cu) would also be important to understanding whether these contaminants pose more of a threat to these native fish than other fish species. Chronic exposures in the lab would also be useful to determine long-term effects, including growth rates and reproductive capacity.

Feeding inanga metal contaminated diets and measuring whole body accumulation could help determine whether diet played a significant role in the accumulation patterns observed in field studies (Chapters 4 & 5). Presenting inanga with a choice between metal contaminated and non-contaminated diets could also provide information on their ability to behaviorally avoid contaminated foods. It is possible that fish in experimental field cages had no choice in terms of food options, so those that accumulated higher trace elements simply had more access to contaminated food. Cu, Fe, and Zn have all been shown to be taken up across the intestine (Glover and Hogstrand, 2002b; Hoyle et al., 2007; Kwong and Niyogi, 2009). Identifying the extent to which trace elements are taken up via the diet and whether or not inanga are able to make choices in terms of food would be an important step to understanding how metals accumulate in inanga.

More work is also needed in determining the specific transporters responsible for cutaneous ion transport, their hormonal control, and the role they may play in challenging environments. Determining the up-regulation of transporters *via* gene expression (Arini et al., 2015; Scott et al., 2004; Yan et al., 2007), immunocytochemical labelling (Hsu et al., 2014; Li et al., 2014), or fluorescent tagging (Dymowska et al., 2014) would help elucidate whether inanga alter the number of these transporters in changing environments. Determining the kinetics of uptake (similar to Glover and Hogstrand, 2002b) in various exposures could also help determine whether transporter affinity changes. Determining rates of cutaneous ion transport in varying environmental factors (salinity, temperature, hypoxia) would also help determine the ecological role of this transport. Changes due to salinity and temperature, for example, may relate to migrational cues; while the ability of

inanga to survive in extreme environments (hypoxia) may be aided by contributions of cutaneous ion transport through compensatory uptake during emersion. It would also be useful to determine whether other galaxiids, like the brown mudfish (*Neochanna apoda*) which inhabits ephemeral ponds (Minns, 1990), are also capable of cutaneous ion transport. This would help establish whether cutaneous ion transport is a trait shared by phylogenetically related fish.

Appendices

Appendix 1. Physicochemical water parameters and fish caught for the 34 streams surveyed along the West Coast of New Zealand's South Island in February 2013.

West Coast Stream	No.	Salinity (‰)	Dissolved Oxygen (mg L ⁻¹)	Temperature (° Celsius)	pH	Land Use	Fish Species Caught
Granity Creek	1	0.7	9.92	15.5	3.9	urban	none
Twins Creek	2	0.1	9.75	15.9	5.1	urban	none
Jones Creek	3	0.1	10.17	16.7	6.5	agriculture	1 bully
Big Ditch	4	0	7.75	17.2	6.1	agriculture	none
Rapid Creek	5	0.1	9.75	16.5	4.5	agriculture	none
Fairdown No. 1	6	0	9.17	16.5	6	agriculture	193 inanga, 9 bullies
Deadman's Creek	7	0	9.52	16.3	6.4	agriculture	1 bully
Omanu Creek	8	0	9.71	17.6	6	natural	14 inanga, 2 bullies, 1 giant kokopu
Island Creek	9	0	9.77	18.1	6.1	natural	2 bullies
9 Mile Creek	10	0	10.22	15.8	6.2	natural	none
10 Mile Creek	11	0	9.87	15.5	6.2	natural	1 bully
Fuschia Creek	12	0	9.96	14.9	6.4	natural	2 koaro
Limestone Creek	13	0.2	10.9	15.1	7	natural	none
Bromielaw Creek	14	0	8.62	21.4	6	natural	1 bully, 1 banded kokopu
Waiwhero Creek	15	0.1	9.36	20.7	6.5	agriculture	none
Mahers Creek	16	0.1	9.28	15.9	6.5	natural	11 banded kokopu
Baker's Creek	17	0	9.63	17.8	6.6	natural	none
9 Mile Creek (Kotorepi)	18	0.2	9.39	19.1	7	natural	none
Coal Creek No. 2	19	0	9.35	20.6	6.2	agriculture	41 inanga
Big Eel Creek (Mahinapua)	20	0	6.94	21.3	7.5	agriculture	1 inanga
Bittern Creek	21	0	6.51	20	5.4	agriculture	53 inanga, 1 longfin eel
Ogilvie Creek	22	0	9.48	16.8	5.6	natural	1 banded kokopu

Appendix 1 (continued). Physicochemical water parameters and fish caught for the 34 streams surveyed along the West Coast of New Zealand's South Island in February 2013.

West Coast Stream	No.	Salinity (‰)	Dissolved Oxygen (mg L ⁻¹)	Temperature (° Celsius)	pH	Land Use	Fish Species Caught
Rocky Creek	23	0	9.2	19.3	5.9	natural	12 banded kokopu
Camp Creek	24	0	7.94	21.2	6.1	agriculture	16 banded kokopu
Woolhouse Creek	25	0	9.2	19.6	6.4	natural	4 banded kokopu, 2 bullies, 1 redfin bully
Horseshoe Bend Creek	26	0	9.31	17.4	6.2	natural	15 banded kokopu
Kokotahi Track Creek	27	0	9.32	18.1	6.2	agriculture	55 inanga, 1 giant kokopu, 2 bullies, 1 redfin bully
Bonar Creek No. 1	28	0	9.6	16.6	6.2	natural	1 banded kokopu
Adamson Creek	29	0	9.09	16.9	6.2	natural	2 kokopu
HSH	30	0	8.77	24.4	5.5	agriculture	12 longfin eels
La Fontain Bridge Creek	31	0	8.87	18.1	7.1	agriculture	none
McCulloch's Creek	32	0	9.41	18.3	5.5	agriculture	none
Culvert	33	0	8.06	21.1	5.4	agriculture	none
Hot Zinger	34	0	7.77	21.9	5.2	agriculture	1 kokopu

Appendix 2. Total water trace element concentrations ($\mu\text{g L}^{-1}$) for the 34 streams surveyed ($n = 1$ each) along the West Coast of New Zealand's South Island in February 2013. Cd and Pb had more than 50% of samples below detection limits and were therefore excluded.

West Coast Stream	no.	Al ($\times 10^2$)	As	Co	Cr	Cu	Fe ($\times 10^2$)	Ni	Zn
Granity Creek	1	299.71	11.14	13.26	17.36	28.88	167.57	187.05	1288.21
Twins Creek	2	78.23	0.15	7.24	0.35	1.99	4.98	36.36	165.19
Jones Creek	3	0.48	0.29	0.05	0.12	0.10	0.05	0.05	1.24
Big Ditch	4	0.78	1.65	0.20	0.13	0.29	3.59	0.18	1.33
Rapid Creek	5	28.03	0.50	6.58	0.51	3.45	21.28	21.01	131.60
Fairdown No. 1	6	2.08	1.52	0.45	0.37	1.14	7.90	0.80	11.00
Deadman's Creek	7	0.97	0.72	0.35	0.21	0.66	9.71	0.75	3.46
Omanu Creek	8	0.53	0.05	0.05	0.05	0.16	0.25	0.05	0.31
Island Creek	9	0.39	0.05	0.05	0.05	0.19	0.03	0.05	0.67
9 Mile Creek	10	0.32	0.05	0.05	0.05	0.11	0.04	0.05	0.38
10 Mile Creek	11	0.24	0.05	0.05	0.05	0.19	0.04	0.05	0.18
Fuschia Creek	12	0.16	0.05	0.05	0.05	0.10	0.03	0.05	0.30
Limestone Creek	13	3.13	0.61	0.36	0.14	0.15	6.40	1.22	5.01
Bromielaw Creek	14	1.40	0.14	0.10	0.20	0.30	4.24	0.20	1.24
Waiwhero Creek	15	0.20	0.39	0.05	0.05	0.44	0.98	0.22	0.55
Mahers Creek	16	0.28	0.34	0.05	0.05	0.32	0.90	0.38	0.67
Baker's Creek	17	0.22	0.44	0.05	0.05	0.15	0.08	0.05	0.80
9 Mile Creek (Kotorepi)	18	23.60	0.49	2.72	0.57	0.57	10.08	7.99	21.47
Coal Creek No. 2	19	0.74	0.25	0.14	0.12	0.22	4.95	0.73	2.01
Big Eel Creek (Mahinapua)	20	1.34	0.13	0.17	0.19	0.29	2.51	0.42	1.91
Bittern Creek	21	5.63	1.33	0.53	0.98	1.17	14.41	0.87	39.41
Ogilvie Creek	22	3.72	0.05	0.12	0.38	0.65	3.45	0.25	2.59

Appendix 2 (continued). Total water trace element concentrations ($\mu\text{g L}^{-1}$) for the 34 streams surveyed ($n = 1$ each) along the West Coast of New Zealand's South Island in February 2013. Cd and Pb had more than 50% of samples below detection limits and were therefore excluded.

West Coast Stream	no.	Al ($\times 10^2$)	As	Co	Cr	Cu	Fe ($\times 10^2$)	Ni	Zn
Rocky Creek	23	3.96	0.05	0.21	0.52	0.80	2.97	0.32	2.07
Camp Creek	24	2.69	0.20	0.05	0.46	0.79	2.61	0.27	2.76
Woolhouse Creek	25	0.72	0.05	0.05	0.05	0.13	0.55	0.05	0.70
Horseshoe Bend Creek	26	2.15	0.05	0.05	0.32	0.29	2.48	0.14	1.46
Kokotahi Track Creek	27	1.95	0.16	0.05	0.33	0.97	3.68	0.12	2.14
Bonar Creek No. 1	28	0.30	0.05	0.05	0.05	0.18	0.12	0.05	2.00
Adamson Creek	29	0.48	0.05	0.05	0.05	0.29	0.94	0.05	0.61
HSH	30	1.33	0.57	0.17	0.12	1.72	1.22	0.34	4.78
La Fontain Bridge Creek	31	0.48	0.49	0.21	0.14	0.17	3.33	0.16	0.67
McCulloch's Creek	32	0.19	1.55	0.05	0.05	0.15	0.09	0.14	0.15
Culvert	33	1.05	0.43	0.41	0.17	0.76	8.27	1.17	4.23
Hot Zinger	34	2.26	0.29	0.05	0.20	0.42	2.07	0.26	1.10

Appendix 3. Total sediment trace element concentrations (mg kg^{-1}) \pm standard error of mean for the 34 streams surveyed ($n = 2$ each) along the West Coast of New Zealand's South Island in February 2013. Cd had more than 50% of samples below detection limits and was therefore excluded.

West Coast Stream	no.	Al ($\times 10^3$)	As	Co	Cr	Cu	Fe ($\times 10^3$)	Ni	Pb	Zn
Granity Creek	1	7 ± 1	31 ± 1	2 ± 1	10 ± 2	4 ± 1	20 ± 7	6 ± 2	7 ± 1	32 ± 10
Twins Creek	2	40 ± 1	4 ± 2	29 ± 1	109 ± 1	51 ± 3	64 ± 3	49 ± 1	13 ± 2	158 ± 4
Jones Creek	3	13 ± 1	2 ± 0	9 ± 1	45 ± 10	9 ± 2	21 ± 2	19 ± 3	15 ± 1	69 ± 4
Big Ditch	4	22 ± 2	20 ± 4	11 ± 0	34 ± 2	22 ± 2	38 ± 2	28 ± 0	19 ± 0	110 ± 2
Rapid Creek	5	12 ± 2	20 ± 4	12 ± 2	20 ± 2	14 ± 2	43 ± 6	25 ± 4	20 ± 1	70 ± 6
Fairdown No. 1	6	5 ± 0	3 ± 0	2 ± 0	6 ± 0	4 ± 0	7 ± 0	5 ± 0	14 ± 1	29 ± 1
Deadman's Creek	7	7 ± 0	4 ± 0	4 ± 0	9 ± 1	7 ± 0	14 ± 0	9 ± 0	18 ± 0	47 ± 1
Omanu Creek	8	16 ± 1	2 ± 0	7 ± 0	19 ± 1	11 ± 1	20 ± 1	11 ± 0	10 ± 0	56 ± 1
Island Creek	9	9 ± 1	0 ± 0	4 ± 1	11 ± 3	5 ± 0	14 ± 2	8 ± 1	7 ± 1	67 ± 9
9 Mile Creek	10	14 ± 1	2 ± 0	7 ± 1	26 ± 7	10 ± 1	21 ± 1	13 ± 3	11 ± 0	85 ± 4
10 Mile Creek	11	21 ± 0	2 ± 0	15 ± 2	58 ± 3	19 ± 1	33 ± 2	32 ± 1	19 ± 1	108 ± 5
Fuschia Creek	12	17 ± 1	3 ± 0	9 ± 1	22 ± 1	18 ± 1	30 ± 1	17 ± 0	20 ± 3	100 ± 2
Limestone Creek	13	16 ± 1	17 ± 0	10 ± 1	34 ± 1	8 ± 0	32 ± 0	20 ± 1	8 ± 0	70 ± 3
Bromielaw Creek	14	8 ± 2	2 ± 0	3 ± 1	15 ± 2	5 ± 1	12 ± 2	7 ± 1	6 ± 2	37 ± 7
Waiwhero Creek	15	29 ± 0	12 ± 1	20 ± 1	47 ± 1	24 ± 1	59 ± 2	56 ± 1	20 ± 2	126 ± 4
Mahers Creek	16	26 ± 0	16 ± 1	21 ± 1	44 ± 1	27 ± 2	61 ± 1	56 ± 0	28 ± 0	142 ± 5
Baker's Creek	17	28 ± 3	20 ± 3	24 ± 1	44 ± 4	56 ± 6	62 ± 3	63 ± 3	52 ± 12	167 ± 2
9 Mile Creek (Kotorepi)	18	16 ± 1	11 ± 1	27 ± 1	35 ± 2	35 ± 0	115 ± 39	58 ± 4	23 ± 3	246 ± 88
Coal Creek No. 2	19	12 ± 2	8 ± 1	9 ± 2	19 ± 4	7 ± 1	25 ± 4	18 ± 3	17 ± 1	70 ± 12
Big Eel Creek (Mahinapua)	20	24 ± 6	18 ± 4	348 ± 76	18 ± 6	26 ± 6	89 ± 21	77 ± 15	33 ± 7	230 ± 55
Bittern Creek	21	18 ± 4	7 ± 3	10 ± 1	32 ± 5	13 ± 1	33 ± 1	28 ± 1	13 ± 0	65 ± 5

Appendix 3 (continued). Total sediment trace element concentrations (mg kg^{-1}) \pm standard error of mean for the 34 streams surveyed ($n = 2$ each) along the West Coast of New Zealand's South Island in February 2013. Cd had more than 50% of samples below detection limits and was therefore excluded.

West Coast Stream	no.	Al ($\times 10^3$)	As	Co	Cr	Cu	Fe ($\times 10^3$)	Ni	Pb	Zn
Ogilvie Creek	22	20 ± 3	5 ± 2	12 ± 2	142 ± 99	8 ± 1	36 ± 3	57 ± 26	12 ± 1	69 ± 15
Rocky Creek	23	4 ± 2	0 ± 0	2 ± 1	4 ± 2	4 ± 1	5 ± 2	2 ± 1	4 ± 1	9 ± 4
Camp Creek	24	25 ± 0	1 ± 0	12 ± 0	47 ± 10	11 ± 1	31 ± 1	28 ± 0	10 ± 1	75 ± 2
Woolhouse Creek	25	48 ± 4	22 ± 19	28 ± 1	104 ± 7	31 ± 2	70 ± 5	62 ± 2	17 ± 0	177 ± 10
Horseshoe Bend Creek	26	32 ± 9	2 ± 1	17 ± 4	69 ± 14	13 ± 0	55 ± 12	39 ± 10	14 ± 1	107 ± 18
Kokotahi Track Creek	27	42 ± 0	3 ± 0	22 ± 1	82 ± 1	22 ± 3	61 ± 4	52 ± 3	20 ± 2	142 ± 6
Bonar Creek No. 1	28	28 ± 2	0 ± 0	17 ± 2	68 ± 1	16 ± 0	45 ± 6	39 ± 3	14 ± 6	114 ± 15
Adamson Creek	29	49 ± 3	3 ± 1	31 ± 2	118 ± 1	46 ± 2	72 ± 5	72 ± 2	19 ± 0	185 ± 6
HSH	30	35 ± 2	9 ± 1	23 ± 4	50 ± 1	40 ± 1	52 ± 4	36 ± 2	43 ± 7	229 ± 13
La Fontain Bridge Creek	31	29 ± 4	4 ± 1	18 ± 3	51 ± 6	23 ± 8	51 ± 5	33 ± 4	11 ± 3	127 ± 13
McCulloch's Creek	32	37 ± 0	8 ± 0	26 ± 0	64 ± 0	42 ± 0	61 ± 1	52 ± 0	5 ± 0	167 ± 1
Culvert	33	27 ± 2	4 ± 1	17 ± 2	45 ± 4	18 ± 1	44 ± 4	29 ± 1	6 ± 2	111 ± 9
Hot Zinger	34	22 ± 2	3 ± 1	20 ± 4	50 ± 10	25 ± 14	42 ± 4	30 ± 8	10 ± 0	96 ± 14

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